THE ENZYMOLYSIS OF CEREAL PENTOSANS

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C O N T E N T S.

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GENERAL INTRODUCTION

In the investigation of any biochemical relationship, it is necessary to gain an understanding firstly of the structure of the substrate itself and secondly of the enzyme systems capable of acting on that substrate. In the present instance the substrate, cereal pentosan, may be classified as a cereal "gum" or water-soluble hemicellulose. Cellulose, the most abundant structural polysaccharide of plants, is generally found in close association with a number of other components such as pectin, lignin and hemicelluloses of varying compositions and properties.

The term "hemicellulose" was first used by Schulze (1891) to describe substances which he isolated from plant cell-walls by extraction with 4% sodium hydroxide which was subsequently neutralised with dilute mineral acids. These substances he believed to be intermediates in the formation of cellulose, but later work showed that, while cellulose contained only glucose, hemicelluloses yielded on hydrolysis primarily pentoses accompanied by hexoses and uronic acids. Hemicelluloses vary in solubility; some, those otherwise termed gums, are soluble in water and others in dilute alkali of differing concentrations; cellulose, on the other hand, is not soluble in sodium hydroxide.

The first reported preparation of a hemicellulose was by Tollens and Stone in 1888 from brewer's spent grains;

it contained xylose and arabinose units. Schulze in 1892 isolated the same two pentoses from the hydrolysates of hemicellulose preparations extracted by 4% sodium hydroxide from wheat and rye. Schulze and Tollens, also in 1892, prepared a xylan hemicellulose from wheat straw and maize stalks. Later, in 1923, O'Dwyer obtained xylose, arabinose, mannose and galactose units from hydrolysates of hemicelluloses obtained from American white oak. The detection of uronic acid residues in some beechwood hemicelluloses by O'Dwyer (1926) and in spent grains by Preece (1931) emphasised the complex nature of the hemicellulosic polysaccharides.

An important advance in fractionation technique was made by Norris and Preece (1930) who employed as source material wheat bran. Pectic substances were removed with ammonium oxalate solution and lignin-content was minimised by treatment with alcoholic soda. The alkaline extract of the residue was acidified to yield a polysaccharide fraction A; addition of half its volume of acetone to the neutral extract caused precipitation of fraction B; addition of a further half volume of acetone yielded fraction C. Each of the fractions was further fractionated from alkaline solution with Fehling's solution to yield hemicelluloses A_1 and B_1

and with Fehling's solution and acetone to yield hemicelluloses A_2, B_2 and C_2. These fractions, precipitated as their copper complexes, were treated with acid and reprecipitated as polysaccharides with acetone. While B_2 consisted substantially of glucan, the other fractions were composed either of pentosans or of uronopentosans.

Such fractionation methods have been subjected to criticism, particularly by Norman (1937) in that the alcoholic soda treatment of the plant tissue cannot be without effect upon the hemicelluloses themselves, especially at high temperatures. This view was later substantiated by Preece (1941). Such procedures have, however, been of great importance in the development of the study of cereal gums.

That two types of hemicellulose could exist in cereal grain was shown by Preece and Hobkirk in 1954; a husk type distributed throughout the grain and containing mainly pentosans (together with uronic acid) was distinguished from a glucan-rich endospermic or reserve type found primarily in the inner endosperm. Solutions of the husk type hemicelluloses had low viscosity compared with those of the endospermic type. It was suggested that the endospermic type hemicellulose acted as a gum, or water-soluble hemicellulose, precursor during the early

stages of growth or during autolysis, a view also proposed by Preece, Aitken and Dick (1953) on the basis of autolysis experiments on different varieties of barley. That the distinction in classification between gums and hemicelluloses was an artificial one depending upon a relatively unimportant solubility character was shown in 1950 by Preece, Ashworth and Hunter when they demonstrated that barley contains in an insoluble form material similar in composition to a water-soluble gum.

The earliest recorded reference to the water-soluble polysaccharides was by O'Sullivan in 1882, when he isolated from barley, wheat and rye what he called "amylans" since he supposed that they were essentially glucans related to starch. He removed low-molecular material from the finely ground grain by exhaustive extraction with alcohol at 40°C. and then extracted with water at the same temperature to obtain a gummy solution. Alcohol was used to precipitate the gum. The crude product was separated into two parts; that soluble in cold water was called β-amylan and that soluble in water at 40°C. α-amylan. The latter he reported to yield on hydrolysis only glucose. The proportion of the two components varied in the cereals examined; in

barley, α-amylan was more abundant (about 2% of the dry grain), whilst in wheat and rye β-amylan predominated.

No further work on the subject of the cereal gums was reported until 1903 when Lindet extracted barley at various stages of germination with water containing mercuric sulphate as an enzyme inactivator and precipitant of nitrogenous compounds. Metallic salts were removed from the extract with baryta and sulphuric acid. Using fractionation with alcohol Lindet obtained a non-cupric-reducing gum, apparently identical with the β-amylan of O'Sullivan, which yielded a mixture of pentoses on hydrolysis. Three years later, in 1906, Brown et al. investigated the water-soluble polysaccharides of barley and malt though their products, owing to the methods of extraction employed, do not necessarily represent materials present in water-soluble form in the original barley. Malt extract was used to liquefy the homogenate made by treating ground barley with boiling water. A crude amylan, precipitated with alcohol, was composed of glucose and pentoses; it was suggested that "amylans" and starch were unrelated. Using a fermentation procedure with yeast, they obtained a substance which yielded arabinose on

hydrolysis and was identical with the "araban" which Wróblewski had isolated from malt in 1897 by aqueous-alcoholic extraction. It is probable that this polysaccharide contained not only arabinose but also xylose (cf. Ford and Peat, 1941).

In 1938, Piratzky and Wiecha prepared extracts of barley and used precipitation with Fehling's solution to isolate a mixture of gums corresponding to the α-amylan of O'Sullivan. They showed that the size of the molecules varied up to a molecular weight of about 65,000. Such products could not, however, be isolated from extracts of malt. As yet, available knowledge on the subject of the water-soluble polysaccharides was generally confusing and scanty but in 1950 the work of Preece et al. made possible some characterisation of the types of molecule involved. To prevent enzymic degradation of soluble or insoluble material, the ground grain was treated with boiling 85% alcohol before aqueous extraction at 40°C. Two gums, named barley gums B_2 and C_2 on the basis of the nomenclature proposed by Norris and Preece (1930) were obtained; hydrolysis showed that C_2, comparable with O'Sullivan's β-amylan, contained arabinose, xylose and glucose while B_2 was essentially of glucan nature. The molecular weight

of the barley gum C_2 was calculated to approximate to 47,500.

Similar lines of investigation were reported by Meredith, Bass and Anderson in 1951 when they described gums from barley, malt and wort of similar compositions to those isolated by Preece et al. (loc. cit.). The yields obtained, however, were approximately three times those reported by Preece et al. since no precautions had been taken to avoid enzyme action during extraction. The necessity for employing an enzyme inactivation procedure was appreciated by the Canadian workers in 1952, when Anderson reported instability of solutions of the gums due to coprecipitation of enzymes. The viscosities of solutions of such gums from barley were later shown by Meredith and Anderson (1955) to be about two hundred times less than corresponding solutions of alcohol-inactivated gum preparations, indicating a large amount of enzymic degradation.

The study of the water-soluble gums was necessarily complicated by the large variety of types of molecule present in any one gum preparation, and a method of selection of different components was sought. Preece and Mackenzie in 1952 introduced what is, so far, the most effective method of fractionation of

gums. Using ammonium sulphate as fractionating agent for aqueous solutions of gums from barley and malt, prepared as described by Preece et al. (1950), they found that increasing concentrations of the salt caused the precipitation first of an almost homogeneous glucan, named β-glucan (then referred to as β-glucosan) and then of pentosan-rich fractions. The mildness of the reagent in comparison with previously suggested alkaline treatments together with the greater efficiency of fractionation commended this method. A preliminary survey of the water-soluble polysaccharides of the cereals wheat, oats, rye and maize showed that oats and maize resembled barley in their richness in glucan material. Whereas, however, the barley gum was laevorotatory, those from oats and maize were dextrorotatory. Wheat and rye, on the other hand, contained higher proportions of laevorotatory pentosan gum which gave rise to viscous aqueous solutions.

A more detailed examination of fractionated gums from the five cereals was performed by Preece and Hobkirk (1953). Fractions were collected from a precipitation level of 20% ammonium sulphate to saturation, at each step the salt concentration being increased by 10%. From barley a good yield of an uncontaminated laevorotatory glucan, β-glucan, was

obtained by precipitation with 20% ammonium sulphate. From rye a reasonable amount of a pure araboxylan was obtained at 40-50% ammonium sulphate concentration. Such araboxylan is substantially absent from maize; oats and barley are poor sources while wheat is fairly rich. This araboxylan from rye has been used as substrate in a large part of the enzymic investigation described below.

Various methods of gum extraction were considered by Meredith et al. (1953) using as source materials barley and malt. The procedure involved primary refluxing of the ground grain with ethanol followed by extraction of the dried grain with either a 1% aqueous solution of papain, dilute hydrochloric acid of pH 1.0 or a 6% (w/v) aqueous solution of trichloroacetic acid (pH 1.0). Products of similar composition but giving rise to solutions of lower viscosity than those prepared using papain were obtained by the two last methods; later (in 1955) the same workers employed in extraction a 0.025% aqueous solution of papain to obtain what they considered to be an undegraded polysaccharide in its naturally-occurring state. Such methods of extraction have been criticised by Preece (1955) who stressed the possibility that the papain may contain enzymes capable

of attacking hemicelluloses; also, the increased yield might be accounted for by aggregation of gum molecules under the influence of the papain, rather than by its inhibition of enzymic degradation.

Investigations of hemicellulose structure, while hampered by the difficulty in obtaining pure polysaccharides, have proved successful in recent years in elucidating the structural features of many such compounds, especially of xylans obtained from lignified tissues. An almost general feature has been found to be a main chain of 1:4-linked β-D-xylopyranose residues. The xylan extracted by alkali from esparto grass has been extensively examined; generally, the products of hydrolysis included L-arabinose (Haworth et al., 1934); the proposal that the xylan chains were terminated at the non-reducing ends by arabinose residues was not acceptable in that the proportion of arabinose residues was not constant but was subject to variation depending on the methods of purification of the xylan. An essentially pure xylan was obtained from esparto grass by Chanda et al. (1950) after repeated precipitations of the hemicellulose with Fehling's solution; investigation of its structure suggested that it contained about 75 xylose residues linked by 1:4-β-linkages and a single

branch point at which the link was 1:3.

The presence of L-arabinose residues in xylans has been the subject of much consideration. Perlin in 1951 isolated from wheat flour a water-soluble polysaccharide which yielded on hydrolysis xylose (63%) and arabinose (37%). The arabinose content in this case could not be altered by fractionation; methylation, followed by hydrolysis, yielded equal amounts of 2:3:5-tri-o-methyl-L-arabofuranose and 2:3-di-o-methyl-D-xylose together with 2-o-methyl-D-xylose and free D-xylose. The structure postulated for the araboxylan was thus a chain of 1:4-β-linked xylose residues carrying single arabinosyl units linked at positions 2 or 3 in the xylose residues.

In general, fractions containing different amounts of arabinose can be separated from crude hemicelluloses obtained from a variety of sources; the mode of linkage of the L-arabofuranose residue to the xylose residue of the chain is usually 1:3. In addition to arabinose, D-glucuronic acid and less frequently 4-o-methyl-D-glucuronic acid have been isolated from the products of hydrolysis of xylans.

Recently, the araboxylan prepared from rye flour by Preece and Hobkirk (1953) was the subject of structural investigation by Aspinall and Sturgeon (1957).

The products of hydrolysis were xylose (60%), arabinose (29%) and glucose (5%) which was considered to be an impurity and not part of the xylan molecule; after methylation followed by hydrolysis the products were 2:3:5-tri-o-methyl-L-arabinose (30%), 2:3-di-o-methyl-D-xylose (36%), 2-o-methyl-D-xylose (31%) and D-xylose (2.5%). Using this and other evidence from periodate studies it was concluded that rye araboxylan formed an essentially linear molecule, containing chains of 1:4-linked β-D-xylopyranose residues with approximately every second xylose residue carrying a terminal L-arabofuranose residue linked through position 3. Thus, this rye pentosan differs from the water-soluble hemicellulose obtained from wheat flour in 1951 by Perlin and in 1955 by Montgomery and Smith in having the majority at least of the arabinose residues linked through carbon atom 3 of singly branched xylose residues, whereas in the wheat pentosan a substantial proportion of arabinose residues are also linked through carbon atom 2 of doubly-branched xylose residues.

There is much confusion in the literature concerning nomenclature of enzyme systems capable of acting on cell-wall materials. Brown and Morris, who observed in 1890 that the barley endosperm cell-walls became degraded during germination, described the systems concerned as

"cyto-hydrolytic" enzymes. The abbreviated term "cytase" is frequently found in the older literature while more specific names such as cellulase, hemicellulase and xylanase are also found. An early investigation on cytase was that of Grüss in 1902 who extracted from green malt an enzyme which hydrolysed galactan in gum tragacanth and mannan from date stones. By mashing together barley and green malt, Baker and Hulton (1917) obtained a larger yield of pentosan than the sum of that in the separate extracts; cytolysis was indicated and it was shown that the active enzyme could be precipitated from extracts by treatment with alcohol. Earlier, Schöne and Tollens (1901) had demonstrated a similar increase in pentosans during the germination of various types of seeds.

Two types of enzyme action were described by Preece and Ashworth (1950): A cytoclastic enzyme action which reduced the molecular complexity of water-soluble non-starchy polysaccharides and, at the same time, increased the solubility of initially insoluble hemicellulosic materials; secondly, a cytolytic enzyme action was characterised by the prolonged production of reducing groups. Later, (in 1954a) a more specific nomenclature was used by Preece et al. to describe enzymes which attack β-glucan. They considered the cytoclastic system to

contain endo-β-glucanase which caused a sharp decrease in solution viscosity without any large development of reducing groups; the cytolytic system embraced both endo- and exo-β-glucanase together with cellobiase action. Such precise nomenclature is favoured the more homogeneous and pure the substrates obtained; interest has been deflected away from mixtures of grain constituents as substrates to comparatively pure components of the grain.

One of the earliest of such investigations was that of Lüers and Volkamer in 1928; they extracted an enzyme from green malt and used as substrates xylans from elder pith and from barley. They identified xylose among the products of hydrolysis and recorded optimum pH (5.0) and temperature (45°C.) for the enzyme; heating at 60°C. for 15 min. caused its inactivation. Some purification of the enzyme was achieved by its adsorption on alumina at pH 5.0 followed by its elution at pH 8.3. A feature of these observations was the extreme slowness of the reaction; even under optimal conditions, 48 hr. were required to achieve 70% hydrolysis.

In 1950 a systematic study of the enzymes involved in the degradation of cell-wall materials was begun by Preece and Ashworth who stressed the necessity for

isolating homogeneous polysaccharides before details of enzyme action could be described. Preece et al. (1950) observed that prolonged contact between the mixed barley gum C_2 and barley enzymes caused a loss in precipitability of the gum. A similar water-soluble polysaccharide named hemicellulose dextrin C_2 was obtained from malt in increased yield and it was deduced that the malt product was at least partially formed other than from the barley gum. Its formation was attributed to the solubilising action of a "cytoclastic" system of enzymes upon the initially insoluble hemicelluloses. In a study of the water-soluble non-starchy polysaccharides during malting, Preece and Ashworth (1950) confirmed the solubilisation of insoluble hemicellulosic material to form material similar to the barley gums, especially fraction C_2. Similar enzymic studies were made by Bass et al. (1953), but again the absence of a homogeneous substrate was a great hindrance.

Determinations of β-glucanase activities were made by Preece and Aitken (1953) and by Preece et al. (1954a) using a modification of a method proposed by Sandegren and Enebo (1952) for "cellulase" determination in barley, the fall in solution viscosity of the substrate being made the basis of assessment of endo-β-glucanase activity.

Diminution in viscosity was accompanied by the rapid production of cellobiose and glucose suggesting the presence of an exo-β-glucanase together with a cellobiase. More recently observations were made by Preece and Hoggan (1956) on the development of the β-glucanase system during malting. In Canada, Bass et al. (1953) used enzyme preparations from barley and green malt with mixed barley gums to demonstrate the presence of endo-enzymes in both sources and of the exo-enzyme at least in green malt. Later, in 1955, this two-fold nature of gum degradation was re-affirmed by Meredith and Anderson again using mixed substrates, and by Bass and Meredith (1955, 1956) using β-glucan.

Artificial substrates have been employed by various workers to assess enzyme activity; thus Ziese in 1931 used hydroxyethylcellulose to demonstrate the presence of "cellulase" in barley malt. Enebo et al. (1953) by considering viscosity changes in solutions of ethyl-hydroxy-ethyl-cellulose under the influence of green malt enzymes concluded that degradation involved not only hydrolytic change but also non-hydrolytic trans-glycosylation. The results obtained using artificial substrates, however, cannot always be compared with those obtained using substrates from natural sources;

Preece and Aitken (1953) showed that artificial substrates are much less sensitive to enzymic attack than are the non-starchy polysaccharides.

Developments in the degradation of pentosans using cereal enzymes have not paralleled those in the glucan field; the main difficulty lies in the contamination of the substrate by glucan but the smaller activity of enzyme preparations is also a drawback. A substantially glucan-free water-soluble pentosan from rye was described by Preece and Hobkirk (1953) who in 1955 used it as substrate in a preliminary investigation of the pentosanases in barley and wheat. Three aspects of enzyme action appeared to be involved: An endo-enzyme causing rapid diminution of viscosity and slow but prolonged liberation of reducing groups with ultimate production of pentose oligosaccharides, including xylobiose; an enzyme responsible for the liberation of free xylose from xylobiose and possibly from other oligosaccharides; an enzyme responsible for the liberation of free arabinose. The low activity of the pentosanase compared with the β-glucanase system of raw barley was stressed. In general, prior information concerned enzymes from malt; following the work of Lüers and Volkamer (1928) who showed the presence of a xylanase in green malt, Lüers and Malsch (1929) showed

that barley contained only 40% of the xylanase activity of its green malt. Using enzymes from raw barley, Bass et al. (1953) demonstrated the production of pentose oligosaccharides from a mixed barley gum.

Several investigations have been made of pentosanase systems in sources other than cereals. Ehrenstein (1926) investigated the decomposition of wheat straw xylan by an enzyme preparation from snails and found that the initial degradation was followed by a much slower decomposition stage. Grassmann et al. (1933a, 1933b) achieved some degree of purification of a xylanase from the mould Aspergillus oryzae by selective adsorption. Enzyme preparations from snails, fungi and barley malt were used by Voss and Butter (1938a, 1938b) to investigate the enzymatic decomposition of xylans from plum stones and beechwood. The kinetics of enzyme action were studied and xylose and uronic acid were detected in the products of hydrolysis. The decomposition of xylan by soil micro-organisms has been extensively investigated by Sørensen (1957). An extracellular xylanase whose excretion was greatly increased during growth on xylan, as compared with non-xylan, carbon sources was capable of random attack on the xylan chain with the production of xylo-oligosaccharides together with mixed oligosaccharides. The extracellular

enzyme was unable to split xylobiose to xylose but a non-extracellular enzyme contained a xylobiase. The degradation of the pentosan from wheat flour by enzymes from fungi, actinomycetes and bacteria has been studied by Simpson (1954); degradation of this substrate by rumen bacteria has been reported by Howard (1957). Xylanase activity has also been reported in the extracts of some seaweeds by Duncan, Manners and Ross (1954, 1956).

In the following report, attention has been confined to cereals as sources of substrates and of enzymes. It cannot be over-emphasised that in such an investigation, extreme caution is necessary in attempting to correlate results from in vitro experiments with processes occurring in the grain itself. Nevertheless, it is not until the actual materials involved have been characterised, in the case of substrates, structurally, and of enzymes, functionally, that the way will be open for the wider consideration of the processes involved in the intact grain, whether during ripening or during germination. It is inevitable that the picture derived from in vitro experiments will be an over-simplified one, but the achievement is undoubted if some understanding of the results of such experiments is possible, even although they must be considered to be merely preliminary.

Chapter 1

AUTOLYSIS RELATIONSHIPS OF BARLEY PENTOSANS

INTRODUCTION

It has, for many years, been an aim of cereal biochemists to derive a relationship between some biochemical property of the barley corn and its malting potentialities. At the termination of modification, as the changes during malting are collectively described, a friable kernel replaces the originally hard barley corn, the change indicating some alteration in the material of the cell-wall of the barley endosperm. The first investigation of this attack on cell-wall material was recorded by Brown and Morris, in 1890; Grüss (1896) and Ling (1904) confirmed their observations, but stressed the incomplete nature of the dissolution. Later, Hopkins and Krause (1937) referred to the action of hemicellulases in the physical changes involved in cell-wall degradation.

The early observation of Piratzky and Wiecha (1938) that the high yield of β-glucan in the earlier stages of malting fell almost to zero in the finished malt was later confirmed by Preece and Mackenzie (1952a). Quantitatively, then, the greatest changes so far observed are those in β-glucan yield, rather than in pentosan yield. Attention was thus drawn to the endo-β-glucanase system of barley, but a survey by Preece *et al.* (1954b)

of a large number of barleys and their behaviour during commercial malting failed to support any relationship between the level of activity of this enzyme system and ease of modification.

Changes in the pentosan content of seeds during germination were examined in 1901 by Schöne and Tollens. Using barley, wheat and peas, a slight increase in pentosan was observed in the germinated seeds. A similar increase in furfurogenic substances during growth was reported by Baker and Hulton (1917), who showed that an enzyme system in green malt was capable of solubilisation of constituents of barley, malt husks and spent grains, with the production of free pentose sugars. Fink and Hartmann (1934), reviewing the problem of pentosan changes during malting, stated that the characteristic physical change from barley to malt was accompanied by an increase in the soluble pentosans of the corn. It was proposed that some link might be possible between modification and pentosan content, but Fink (1935) later concluded that such a connection was unlikely, since the recovery of soluble pentosan became constant several days before modification was complete, a synthetic mechanism presumably balancing the degradative process.

It is, nevertheless, certain that the changes during modification reflect to some degree the conversion

of insoluble to soluble hemicellulosic material, and the simultaneous degradation of the latter. With a view to an overall assessment of these two factors, an autolysis technique was used by Preece and Mackenzie (1953) and by Preece and Aitken (1953), in a comparative study of several samples of different varieties of barley. Preece and Aitken (loc. cit.) and Preece et al. (1954b) concluded from their observations that autolytic behaviour could be related to extremes of malting behaviour, the gum recovery from the better malting barleys quickly rising to a maximum before falling. Gum recovery in the early stages of autolysis was obviously not only governed by the enzymic potentialities of the grain, but also by mechanical penetration of the grist by the solvent. These results were broadly confirmed by van Roey and Hupé (1955), also using an autolytic method.

In connection with such studies, it is important to bear in mind that the balance of enzymes, rather than the actual activity of any one enzyme, will be the governing factor. Also, with β-glucanase activity normally outstandingly greater than pentosanase activity, the rate of degradation of β-glucan would be expected to be the predominant factor in the process, thus obscuring changes in pentosan. A further disadvantage of the method is its inability to take account of the

possible enhancement of enzymic activity during malting. That such a capacity exists within the grain is undoubted for several enzyme systems; Preece and Hoggan (1956) observed that the endo-β-glucanase activity of finished malt from Ymer barley was about one hundred times greater than that of the ungerminated cereal; exo-activity also increased during growth, though it was almost eliminated on the kiln. It is clear, then, as pointed out by van Roey and Hupé (loc. cit.), that high gum content may be no disadvantage in malting, provided suitable enzyme systems for gum degradation are present, or can be produced during germination.

The autolytic method has been applied only to soluble hemicelluloses (or gums); that insoluble hemicelluloses, too, may be of importance in determining the ease of modification of a particular sample of barley has been suggested by Massart and van Sumere (1955). Using 4% sodium hydroxide solution as solvent for water-insoluble hemicelluloses, they obtained two precipitates, namely S_1 (precipitated from the alkaline solution by Fehling's solution alone) and S_2 (precipitated by Fehling's solution in the presence of acetone). The relation $[100(2S_1 + S_2)]$ was found to be linked to the malting quality of the barley, as assessed by an independent expert, in that those barleys with the

lowest index malted the most readily. While this result was based on a relatively small number of samples, it is of significance, if only in that it directs attention from the water-soluble to the water-insoluble components of the grain.

The insoluble hemicelluloses of barley have frequently been referred to as the precursors of malt gum [see, e.g. Preece and Hobkirk, (1954)]; direct evidence in support of this view has recently been advanced by Preece and Hoggan (1957), as a result of investigations of carbohydrate modification during malting; evidence, from a consideration of both water- and alkali-soluble hemicelluloses, suggested that the solubilization of β-glucan was a predominantly enzymic effect, with only a small contribution by mechanical factors. On the other hand, mechanical, rather than enzymic, factors seemed prevalent in pentosan solubilization. The present investigations provide further evidence to substantiate these findings, at least in the case of the ungerminated grain.

EXPERIMENTAL

Water-soluble hemicelluloses (gums) - The method employed was that described by Preece and Aitken (1953),

the equivalent of 25 g. dry weight of a finely-ground, Scottish-grown, Ymer barley of the 1955 harvest being used in each experiment; periods of autolysis of 15, 30, 60, 120 and 240 min. were applied. The compositions of the products were determined as described below, and since glucan material may consist of α- or β-linked type, the results, which are recorded in Table I, refer only to the pentosan components of the gum. While araban and xylan are given separately, there is no doubt that much of the "araban" is combined as araboxylan on the Perlin (1951) model [see also Aspinall and Sturgeon, (1957)].

Autoclave-soluble materials - The grain residue after autolysis was at once treated with boiling water, and destarched according to the method of Preece and Hobkirk (1954), a total of 0.4 g. of a malt α-amylase preparation per 100 g. of original grain being used, during 3 to 4 treatments. Fehling's solution and acetone were used to precipitate hemicellulosic components, which were found to be associated with about 15% of non-carbohydrate material. Comparison of the pentosan components of the autoclave-soluble material and the malt α-amylase preparation indicated that substantially all of the pentosan could be accounted for by the enzyme. In relation to the destarching treatment, it has been shown (Preece and Hoggan, 1957) that such α-amylase treatment

TABLE I

PENTOSAN AUTOLYSIS PATTERNS OF YMER BARLEY

Fraction	Anhydro-sugar units	Yield (mg. per 100 g. barley) after autolysis for:				
		15 min.	30 min.	60 min.	120 min.	240 min.
Water-soluble (40°C.) gum	Araban	91	150	197	173	242
	Xylan	121	188	222	259	332
NaOH-soluble, S_1	Araban	376	419	516	436	425
	Xylan	883	1,225	1,140	1,065	1,101
NaOH-soluble, S_2	Araban	173	226	232	299	245
	Xylan	238	170	232	276	267
Totals	Araban	640	795	945	908	912
	Xylan	1,242	1,583	1,594	1,600	1,700

has no significant influence on the water-soluble non-starchy polysaccharides.

Alkali-soluble hemicelluloses - Hemicelluloses were extracted during 3 treatments with 4% sodium hydroxide according to the method of Preece and Hobkirk (loc. cit.). Upon the addition of Fehling's solution to each water-bright extract, a gelatinous precipitate was obtained (S_1 precipitate); a fibrous precipitate was obtained from the mother-liquor upon the addition of acetone (S_2 precipitate). The results for pentosan composition of the hemicelluloses S_1 and S_2 are also shown in Table I.

Action of isolated enzyme preparations - (i) Preparation of enzymes - Enzymes were prepared using the barley described above and green malt (not derived from the above barley), according to the method outlined by Preece et al. (1954a), the material which precipitated upon the addition of 4 volumes of acetone being collected. The amount of enzyme corresponding to 25 g. dry-weight of original grain was calculated, care having been taken during both preparations to ensure that yields were comparable. It must be stressed, however, that necessary losses during the preparation of the enzyme may be appreciable, resulting in the use of a smaller amount of enzyme than the true equivalent of 25 g. (ii) Preparation of substrate and its incubation with enzyme -

Barley was destarched as above and the grain thoroughly washed. The equivalent of 25 g. raw grain was used as substrate. This was suspended in 200 ml. water; 5 ml. of phosphate-citrate buffer (Britton, 1942) of pH 5.0 was added, followed by the corresponding amount of enzyme dissolved in 100 ml. water. Controls without enzyme (substrate blank) and without substrate (enzyme blank) were incubated at the same time. Incubations were made at 25°C. for 4 hr. and 24 hr., using the barley and green malt enzymes. At the end of incubation, the residual grain was separated by centrifugation and the extract collected and treated with Fehling's solution and acetone in order to precipitate any polysaccharide material. The controls were similarly treated. The results are shown in Table II.

(iii) Examination of conversions for sugars and oligosaccharides - In a series of parallel experiments, the mother liquors after the appropriate incubation periods were heated at 100°C. for 3 min. in order to inactivate enzymes. After cooling and the addition of 2 volumes of ethanol, the mixtures were allowed to stand in a refrigerator overnight, to permit maximum precipitation of high-molecular material. Following filtration through kieselguhr, the clear alcoholic solutions were evaporated to dryness and the residues

TABLE II

ACTION OF GRAIN ENZYMES ON DE-STARCHED BARLEY RESIDUES

Enzyme	Barley		Green malt		nil	
Time of action (hr.)	4	24	4	24	4	24
Polysaccharide units:						
Araban*	8	∕	72	20	0	6
Xylan*	12	∕	96	20	0	6
In mother liquor≠						
Arabinose	-	-	-	++	-	-
Xylose	-	-	-	++	-	-
Xylobiose	-	-	-	+	-	-
Higher pentose oligosaccharides	-	-	+	+	-	-

* Mg. per 100 g. of original grain, and corrected for increment due to enzyme.

∕ Yield positive, but too small for manipulation.

≠ By paper chromatography, and corrected for products of enzyme autolysis.

dissolved in 30% ethanol; chromatograms were prepared and run in the upper layer of the solvent butanol-acetic acid-water (40:10:50). The chromatograms were sprayed with aniline oxalate, which allows distinction between pentose and hexose sugars and oligosaccharides. The results are also shown in Table II.

Action of enzyme preparations on soluble cereal pentosan - Using typical incubation mixtures as described in chapter 4, the above barley and green malt enzymes were shown to attack slowly rye arabo-xylan, with the production of arabinose, xylobiose and a pentose trisaccharide, together with small amounts of higher oligosaccharides and a trace of xylose.

Preparation of malt α-amylase - 100g. malt was ground and extracted at room temperature with 500 ml. water for 1 hr. To the filtered, autolysed solution, 0.2 g. calcium acetate per 100 ml. extract was added, and the temperature raised to 70°C. for 15 min. The solution was dialysed against running water for 3 days, filtered and the enzyme precipitated with 3 volumes of acetone. It was dried slowly. The yield per 100 g. malt was generally from 1.0 to 1.5 g. A portion of the enzyme was hydrolysed and its percentage carbohydrate and sugar composition determined as described below. In 0.40 g., the quantity of enzyme used in destarching 100 g. barley,

0.039 g. was of glucan, 0.055 g. of araban and 0.090 g. of xylan nature, the percentage carbohydrate associated with the enzyme being 46%.

Quantitative examination of hemicelluloses - 20 mg. of the sample was hydrolysed by refluxing with 20 ml. **N** sulphuric acid for 4 hr. The cooled hydrolysate was neutralised with **N** sodium hydroxide solution using methyl red as indicator; 1 drop of **N** sulphuric acid was then added, the acid being preferred to the alkaline side of the end-point on account of the possibility of Lobry de Bruyn transformation occurring during concentration. Sodium sulphate formed was precipitated on the addition of 4 volumes of ethanol. To ensure as complete precipitation of the salt as possible, the mixture was left for 1 to 2 hr. in the refrigerator before filtration. This was found to reduce interference by salts in the separation of sugars on chromatograms. The filtrate was evaporated to dryness for re-extraction with a minimum of solvent. The method described by MacLeod (1951) using paper partition chromatography in conjunction with the Somogyi (1945) method was employed for the estimation of the component sugars. The chromatograms were run on Whatman No. 3 MM paper and were irrigated for 4 days with the upper layer of the system butanol-ethanol-water (45:5:50), this solvent effecting a satisfactory separation of arabinose and

xylose.

Determination of percentage carbohydrate recovery - Preece and Aitken (1953) observed that, in autolysis experiments, recovery of carbohydrate represented from 95-100% of the crude gum; for water- and alkali-soluble hemicelluloses the results are based on 100% recovery of polysaccharide. For autoclave-soluble materials, however, the percentage carbohydrate recovery was ascertained as follows: The neutralised hydrolysate (about 40 ml.), prepared as described in the previous section, was made up to 50 ml. in a graduated flask with distilled water. 5 ml. aliquots were withdrawn, and the usual Somogyi (1945) micro-method applied. The results were calculated in terms of glucose equivalents. The remainder of the hydrolysate was then treated as above, and the component sugars estimated quantitatively.

Specimen calculation of percentage carbohydrate recovery -

Sugar	Conversion factors	Anhydro-sugar	ml. 0.005N $Na_2S_2O_3$	mg. poly-saccharide
Glucose	0.14	0.126	18.64	18.64x0.126
Arabinose	0.16	0.141	0.48	0.48x0.141
Xylose	0.14	0.123	0.92	0.92x0.123
			20.04	

Total glucose equivalent (for 21 mg. gum) = 143.9 ml.

glucan = $143.9 \times \frac{18.64}{20.04} \times 0.126$ = 16.86 mg.

$$\text{araban} = 143.9 \times \frac{0.48}{20.04} \times 0.141 = 0.81 \text{ mg.}$$

$$\text{xylan} = 143.9 \times \frac{0.92}{20.04} \times 0.123 = 0.48 \text{ mg.}$$

Total polysaccharide = 18.15 mg.

$$\text{Hence percentage recovery} = \frac{18.15}{21} \times 100 = 86.5\%$$

DISCUSSION

Firstly, let us consider the present results in the light of previous investigations. The survey by Preece et al. (1954b) of the autolysis patterns of a large number of barleys indicated that those which gave an initial steep rise to the gum maximum, followed by a decrease after about 2 hr. of autolysis, were the best malting samples; Fig. 1, which shows the pattern for the total water-soluble gum in the present Ymer sample, also shows the patterns of total pentosan (as recorded in Table I) and of total glucan (not quoted in the Table). A maximum of water-soluble gum is, indeed, reached early in the 4 hr. period, but the desirable fall towards the end of autolysis is not evident. While Fig. 1 stresses the influence of glucan on the autolysis curve, the pattern

Figure 1.

Figure 1.

Autolysis pattern for a sample of Ymer barley.

T, total water-soluble materials.

G, water-soluble glucan.

P, water-soluble pentosan.

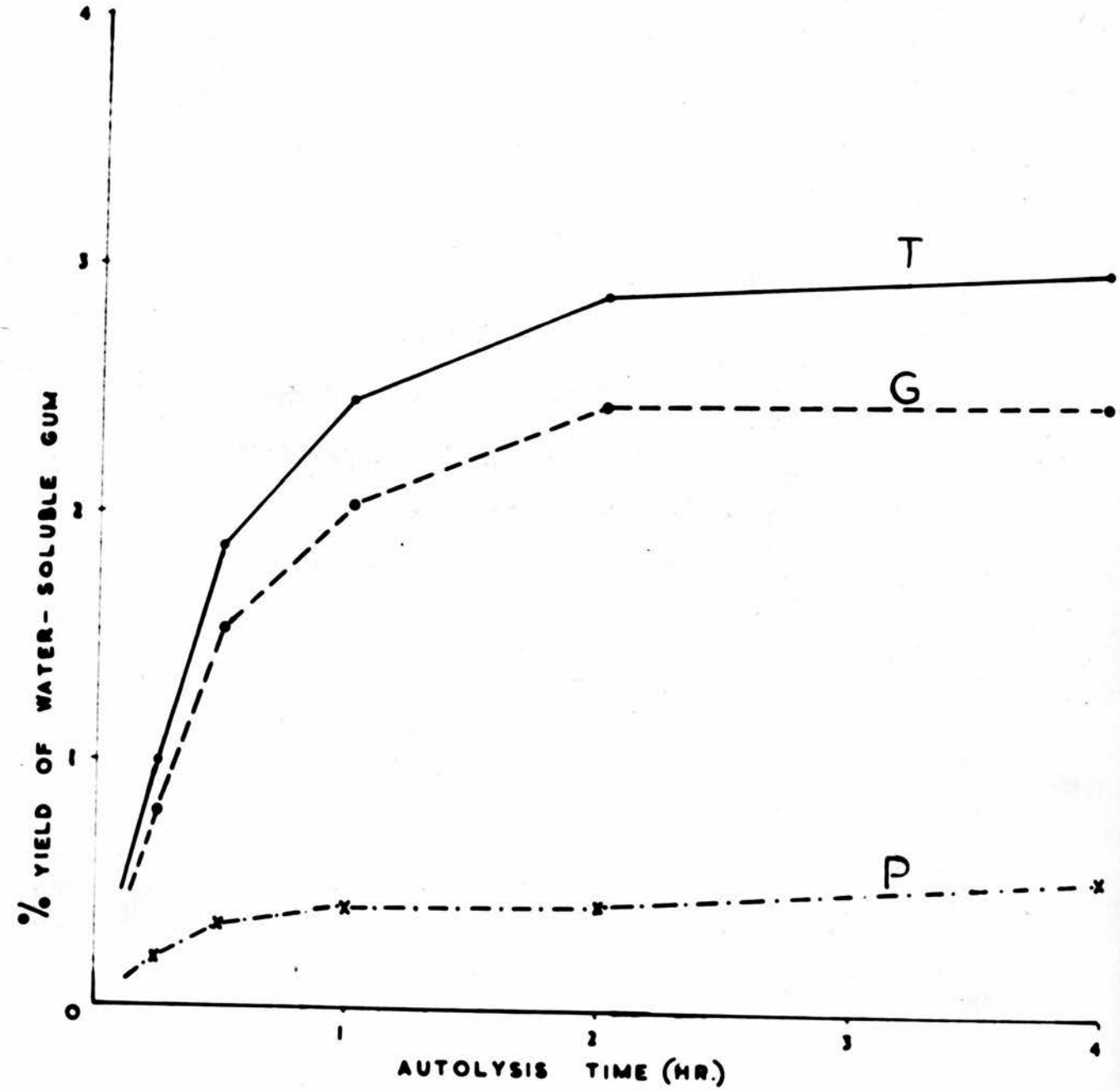


FIG. 1

of soluble pentosan present in the gum is seen to be characterized by a gradual but steady increase, the final yield recorded being about three times that at 15 min. It should be noted, however, that with some of the barleys examined earlier by Preece and Aitken (1953) there was a tendency for the total soluble pentosan to decrease towards the end of the 4 hr. period.

An unusual characteristic of this barley is its possession of initially insoluble hemicelluloses capable of fractionation by Fehling's solution to yield the two fractions S_1 and S_2, distinct not only in solubility, but also in araban/xylan ratio. This ratio approximates to 30/70 for S_1 and to 50/50 for S_2 which, in this respect, therefore, is more akin to the water-soluble gum (for which the ratio is nearer 45/55) than to S_1. This capacity for fractionation may, indeed, be unusual for the hemicelluloses of British-grown barleys, but it has been observed previously with a Californian and a Cyprian barley (Preece et al., 1950) and with several Belgian barleys (Massart and van Sumere, 1955). According to the Belgian authors, the S_1 fraction has a predominant effect on malting quality; thus, on this account also, the present Ymer sample would fail to reach the higher quality level amongst British malting barleys.

It would be most desirable to be able, from the above

results, to trace the transitions undergone by the different types of hemicellulose during autolysis, but this is, at present, impossible, in view of the large number of factors to be considered. There is the possibility of solubilization of initially insoluble hemicelluloses, whether by enzymic or other means; there is likewise to be considered the degradation of water-soluble gums to material irrecoverable by the precipitation method employed. A further complicating factor is the impossibility of distinguishing at present between araban of the pectic type, which almost certainly occurs to some extent in cereals, and araban linked with xylan, in an araboxylan molecule, as characterized by Perlin (1951).

Some general statements, however, are possible. The araban figures show a tendency for water-soluble araban to increase steadily, while total araban is virtually constant after 1 hr. Similarly, there is a steady increase in water-soluble xylan, and initially insoluble xylan is approximately constant after the first half-hour. There is a suggestion of transition from fraction S_1 to water-soluble gum *via* fraction S_2, but the above results do not provide conclusive evidence of such a mechanism, since degradation of the gum may well be balanced by further solution of initially insoluble hemicelluloses as S_1 material.

Enzymic solubilization - The results of Table I do not

allow clear judgement as to whether or not enzymic solubilization of initially insoluble hemicelluloses occurs, but some deductions in this direction may be made from Table II. There is a slow dissolution of hemicellulose caused by mechanical penetration by the solvent, and whether this results from incomplete washing of the grain residue prior to enzyme action is without consequence to the present argument; certainly, no material is rendered soluble in 4 hr. in the absence of the barley enzyme, whereas a measurable quantity is produced when the enzyme is present; in 24 hr., most of this small amount of material has undergone degradation, although the molecules produced are still too large to be detected chromatographically. The picture is similar, but more pronounced, with the green malt enzymes; detection of higher oligosaccharides on a paper chromatogram is possible, even at 4 hr., and arabinose, xylose and xylobiose are produced in appreciable quantities in 24 hr.

These observations relate directly to the results of the autolysis experiment; the pentosan pattern would not be expected to undergo pronounced changes, for no pronounced degradation of water-soluble material in 4 hr. is evidenced by the chromatographic investigation, and enzymic solubilization would not be

without significance in the observed slight, but steady, increase in water-soluble material.

Mechanism of solubilization - The most striking feature of the results of Table II is, perhaps, the extremely low activity of the enzymes from ungerminated barley. It is certain that this lack of activity is partly due to the lower ratio of enzyme to substrate in the *in vitro* experiments as compared with that in the grain in its natural state, but it is improbable that this factor would, of itself, account for the observed results. Although no means is yet available for measuring pentosanase activity directly in grain extracts, there is little reason to suppose that losses, whether due to mechanical factors or to denaturation of the enzymes, would exceed those measured for endo-β-glucanase, for which recovery was found to be 40-70% of the initial activity detectable in extracts (Preece and Hoggan, 1956).

At the end of the 4-hr. period, the pentosan recovered in a soluble state represents only 0.02% of the original barley; it accounts for less than 1% of the hemicellulose soluble in 4% NaOH, and for an even smaller percentage of the total residual hemicellulose. If the material solubilized were at once enzymically degraded, detection should be possible in the chromatographic examination, as it is, in fact, in the presence of the enzymes from green malt. It must,

therefore, be concluded that the capacity of the barley enzyme for solubilization is extremely small. Material present in the soluble form must be subject to slow enzymic degradation, but the 24-hr. period, with the amount of enzyme available, is not sufficient to yield the products observed by Preece and Hobkirk (1955) using a very large concentration of enzyme. These products were, indeed, obtained, as shown in Table II, after the 24-hr. incubation period with the enzyme preparation from green malt, which liberated chromatographically detectable oligosaccharides even in 4 hr.

As was previously suggested by Preece et al. (1954b) - although less significance was then attached to the idea than is now thought to be appropriate - the ease of penetration of the tissue by the solvent may be the major factor responsible for solubilization in the autolysing grain. Penetration will, at all times, be influenced by materials of carbohydrate or other nature present in the grain. For example, the gradual disruption of β-glucan would facilitate indirectly attack on other materials, such as pentosan, which may be present; even during the 4-hr. autolysis period, the β-glucan in many barleys - and this seems to be characteristic of the better malting barleys - undergoes significant degradation. Apart from hemicellulosic

material, however, there may be other components of the grain which hinder penetration. The most likely is protein. It has been observed (Meredith and Anderson, 1955; Meredith et al., 1955) that the presence of papain during the water extraction of gum from barley allows an increase in gum yield, this increase being of significance, not only in the case of β-glucan, but also, though to a smaller degree, in the case of pentosan, the yield of which is augmented by some 50%. It was suggested by the above workers that the action of the papain was to inactivate enzymes capable of attacking the gums, but it is not inconceivable that, instead of this effect, or accompanying it, the papain may have a less specific proteolytic effect; thus, by degrading protein, the papain would facilitate the access of the solvent to the polysaccharides. The general picture of the process of solubilization would thus be a complex one; penetration by the solvent would permit enzyme action, particularly perhaps upon β-glucan and protein; this, in turn, would favour further penetration by the solvent. It is of interest to note that a similar conclusion has been reached by MacLeod and McCorquodale (1958) from microscopic studies of cell-wall disorganization in the endospermic region of different barleys and of seeds from species of the grass Bromus. With B. mollis and

most of the barley samples examined, separation of the cells was found to occur only when papain was present in the surrounding medium. An intercellular matrix of protein was thus envisaged, removal of which was necessary before cell separation could take place.

Further evidence for the predominant penetration, as opposed to the enzymic, factor responsible for pentosan solubilization is provided by the work of Preece and Hoggan (1957), from studies of carbohydrate modification during malting. Water-soluble pentosan was found to increase slightly during the conversion of barley to malt, whilst solubilization of pentosans during kilning was extensive. A purely mechanical action was considered to be responsible for solubilization, at least in the early stages of malting, although the subsequent development of pentosanase activity was probable; this is in agreement with the observed activity of the green malt enzyme used above. The solubilization of β-glucan, on the other hand, appeared to be a predominantly enzymic effect, with only a small contribution by mechanical factors.

In addition to the production of water-soluble materials, however, there are other changes to be considered, as, for example, in the amounts of the hemicellulose fractions in the autolysis experiment. It might be expected that the sodium hydroxide would,

of itself, possess high penetrative power, but there is little doubt that solubilization of the type suggested above would facilitate its penetration. The regularities of the araban/xylan ratios in the barley S_1 fractions (30/70), in the water-soluble fractions (45/55), and (after the first half-hour) in the S_2 fractions (approx. 50/50) are of particular interest. In the original grain, the compositions of the water-soluble and S_2 fractions are substantially identical, and it seems that solubilization of S_2, if it occurs, is not accompanied by a substantial change in relative composition; solubilization is, therefore, either purely mechanical, or is dependent on scission of the xylan back-bone chains. The evidence already discussed suggests that both factors operate; the mechanical factor is predominant in the raw grain, while the enzymic change may become significant as malting proceeds. On the other hand, conversion of S_1 to S_2 would require a more profound change, and trans-arabinosylation immediately suggests itself as a possible mechanism; Preece and Hobkirk (1954, 1955) have already presented evidence for this mechanism in the case of wheat. An enzymic factor may, therefore, be responsible for the small changes observed in the yields of hemicellulose soluble in 4% NaOH.

Practical significance - From the point of view of the maltster, the above conclusions amply confirm the already well-accepted view that the modification of the barley grain, in the early stages of malting, is dependent as much on its gross composition and initial physical condition as on its enzymic potentialities. Changes in pentosan, while small, are not necessarily unimportant, for the increase in soluble pentosan from barley to malt is certainly reflected in changes in wort composition, and, therefore, in properties. The salient feature of these and other observations, however, is the improbability of finding a link between any one set of barley components and ease of modification; numerous factors operate, and a complex interplay of these will determine whether or not modification will proceed smoothly. Any initial deficiencies in the grain may well be balanced out as the enzymic systems develop during malting; but it would appear that the early stages may be of special importance, for here, namely, in the steep, conditions will determine the future course of germination. It is in these initial stages that the factors discussed above operate most strongly, and it may be suggested that - from the practical point of view - more consideration of the conditions during steeping would be rewarding in

controlling the subsequent development of the grain.

SUMMARY

1. An autolysis technique has been applied to a sample of Ymer barley; while the pattern of behaviour of water-soluble pentosans is obscured by the comparatively large changes in β-glucan, an increase in the amounts of soluble pentosans is evident, during the 4-hr. autolysis period applied.

2. Some solubilization of initially insoluble hemicelluloses is observed, and it would appear that mechanical, rather than enzymic, factors play an important part in pentosan solubilization.

3. Ease of penetration of the tissue by the solvent is of major importance and this is probably governed, to a large extent, by the presence of other materials, such as β-glucan and protein.

4. The influence of enzymes on non-pentosan components may control the availability of pentosan for dissolution, both in water and in 4% sodium hydroxide.

5. The experiments point to the early days of malting as the most critical in controlling subsequent modification, and emphasize the importance which should be attached to the physical condition of the grain.

Chapter 2

THE NATURE OF THE GLUCAN CONTAMINATION OF RYE ARABOXYLAN

INTRODUCTION

The constituent units of the water-soluble gum-like polysaccharides in cereals appear to be essentially the same amongst the five common cereals, namely glucan, xylan and araban with small amounts of galactan and mannan. While the mode of association of these sugar units has received consideration, few conclusive results have been obtained; it is not yet known to what extent the araboxylan, which is undoubtedly present, may be accompanied, in crude preparations, by other molecules containing the residues concerned, and particularly by free araban. Perlin (1951) expressed the view that in wheat flour the probable structure of the major pentosan component is one containing a chain of anhydro-D-xylopyranose units joined together by 1:4-β-links; attached to the anhydroxylose units at the 2- or 3- positions are anhydro-L-arabofuranose residues. It seems reasonable to assume, however, that while such a structure might well exist, a mixture of all three types is present in the crude gum, fractionation producing a selection of one or other form.

Prior to a study of the enzymes responsible for the breakdown of cereal pentosan, it was desirable to prepare a pure pentosan substrate free from glucan.

Preece and Hobkirk (1953) stated that glucan present in essentially pure pentosan was a concomitant and not part of the pentosan molecule. They reported the isolation of pure arabo-xylan from rye but the particular sample used was probably atypical in its low glucan content; all later products prepared in the same way contained glucan.

Of the glucan present in the gum-like material from cereals two types have been recognised. Firstly, β-glucan is a fibrous, water-soluble polysaccharide yielding only glucose on hydrolysis. In solution, it has a high viscosity and is laevorotatory. It may be obtained in a virtually pure condition from barley by fractionation with 20% ammonium sulphate (Preece and Hobkirk, loc. cit.). It has been proposed (Aspinall and Telfer, 1954) that the β-glucan molecule is essentially linear with approximately equal numbers of 1:3- and 1:4-β-glucosidic linkages. Secondly, the term α-glucan includes the most abundant non-cellulosic glucose-containing material in plants, namely starch. Starch is commonly regarded as insoluble in water; it is dextrorotatory and is largely constituted with 1:4-α-linkages, but contains also about 5% of 1:6-α-linkages. Apart from what would normally be considered to be a starch-like molecule, a small proportion of related material

possessing solubility in water and probably consisting of molecules of shorter length and dextrinous nature may also be present. Whether such molecules would be present naturally in the grain or would result from starch by mechanical treatment such as grinding or by slight enzyme action before complete inactivation during the initial alcohol treatment (Preece and Mackenzie, 1952a) is unknown. The influence of milling on starch has been extensively investigated by Lampitt et al. (1941, 1947, 1948); Preece and Mackenzie (1952b) showed that such treatment increased the amount of water-soluble polysaccharides and suggested that such an increase represented not only the production of soluble starch or of dextrins, but also, perhaps, some modification of previously insoluble pentosans. Abrasive treatment in certain types of mill has, in fact, been found to reduce appreciably the viscosity of the water-soluble gums, particularly β-glucan, normally extracted (Hoggan, 1957).

Apart from the iodine colour-test which gives the characteristic blue colouration when starch is present, and a pale green colouration with what is considered to be pure β-glucan, specific rotations ($+140^{\circ}$ to $+160^{\circ}$ for dextrinous starchy material and at least -12° for laevorotatory gum) may also indicate the type of glucan present.

Starch itself is a mixture of two components called amylose and amylopectin. Amylose which is generally considered to average about 20% of the whole starch gives a deep blue colour with iodine; amylopectin produces viscous aqueous solutions and gives a purple-red colour with iodine. The constitution of amylose is thought to be one in which α-D-glucopyranose units are linked together as in maltose forming very long chains of perhaps 300 glucose units, the molecular weight being in the region of 50,000 (Percival, 1953). Although amylose is an essentially unbranched molecule, it is branched to a small degree, at least in some preparations, as is shown by the fact that the enzyme β-amylase which occurs in plants (e.g. wheat, barley and soya bean) hydrolyses amylose, with the production of maltose, to an extent of 70-100%, the enzyme being unable to split or pass an as yet unidentified linkage towards the chain end.

In amylopectin, a "repeating unit" of 20 glucose units was found to be present, i.e. for one non-reducing end-group there were 20 glucose residues. To account for the larger molecular size suggested by physical measurements, Haworth et al. (1937) suggested that the basic chains were joined by 1:6-links to form a "laminated", three-dimensional structure by the union of a series of such chains. Among other proposed

formulae there is the "ramifying" or multibranched structure of Meyer and Bernfeld (1940). β-amylase is capable of hydrolysing only 1:4-α-links with the removal of maltose, a 1:6-link being left unattacked. Amylopectin gives maltose (about 50%) on treatment with β-amylase leaving unattacked a molecule of high molecular weight known as a β-dextrin; the exterior chains in this molecule contain only 2 or 3 glucose residues.

The enzyme known as α-amylase which occurs in salivary and pancreatic secretions and in barley malt as well as in some bacterial and fungal extracts is capable of random hydrolysis; while unable to split the 1:6-link, it can bypass such a link; viscosity and iodine-staining power of the substrate rapidly decrease. Firstly, α-dextrins containing 6 to 10 glucose residues are formed, those from amylose being linear and from amylopectin branched. Further action of α-amylase breaks down α-dextrins to reducing sugars. The mode of action as well as the optimum conditions of different α-amylases is distinctive; whereas the optimum temperature for the action of malt α-amylase is 65°C., that for salivary and pancreatic amylase is nearer 37°C. Malt-α-amylase, unlike the salivary enzyme, is readily capable of hydrolysing the linkage adjacent to a reducing group, thereby liberating glucose.

It is certain that glucan of both α- and β-linked types occurs to a greater or lesser extent in all cereals; the actual amounts of each will be dependent not only on the cereal, but also on the particular sample concerned. Some knowledge, in particular in the case of rye, of the relative amounts of the two types of glucan was desirable, and their susceptibility, or otherwise, to α-amylase treatment was made the basis of distinction between them. β-glucan is unattacked by this enzyme, while starch yields a variety of molecules of comparatively low molecular weight.

The actual products of amylase treatment were not investigated in the work under review, as the principal importance of amylase treatment was to remove starch contamination, if present, as completely as possible from rye pentosan. For this purpose, the method of dialysis against running water was employed after α-amylase action; this method is, of course, limited by the size of the molecule, but if α-amylase action completed its normal course, complete removal of the end-products should be effected by dialysis. The action of β-amylase would thus be insufficient since β-dextrins of high molecular weight from amylopectin would not be capable of removal by dialysis.

If, after exhaustive amylase treatment of the pentosan material, glucan were still present, it must

be concluded that it is of the β-linked type which is not capable of easy removal since no specific enzyme free from pentosanase action can yet be prepared.

EXPERIMENTAL

Three sources of α-amylase were used, namely saliva, commercial pancreatin and malt. Usually a preliminary experiment was performed on a small sample of araboxylan; this proving satisfactory, about a kilogram of rye was extracted and treated with α-amylase.

Preparation of water extract - All water extracts of rye were made according to the method of Preece and Mackenzie (1952a) and followed alcohol inactivation of the ground cereal.

Preparation of α-amylase

Salivary α-amylase: Saliva was shaken with four times its volume of alcohol and the α-amylase precipitate was removed by centrifugation; a suspension was made by grinding the precipitate with water in a mortar. A chromatogram to show the products of hydrolysis with N sulphuric acid of salivary amylase showed the presence

of ribose, derived from the enzyme preparation itself.

Pancreatic α-amylase: The method is based on that described by Meyer et al. (1947). Crude pancreatin, which on hydrolysis yielded large amounts of glucose, was extracted for 12 hr. at about 5°C., with occasional stirring, with 0.5N sodium acetate solution. Insoluble material was removed by centrifugation. A small portion of the extract was boiled, cooled and added to the remainder of the extract which was then dialysed for 2 days. This procedure has been reported (Meyer et al., loc. cit.) to delay enzyme inactivation during dialysis. Material which had separated out was removed by centrifugation and the α-amylase preparation was precipitated by the addition of 3 volumes of acetone. It was carefully dried. Only a trace of ribose was detected. For normal purposes it is unnecessary to isolate the dry solid enzyme; the acetone-precipitated enzyme may be dissolved in water and this solution used.

Malt α-amylase: 100 g. malt was ground and extracted with 500 ml. water for 1 hr. at room temperature with stirring. The extract was filtered bright through fluted filter paper and allowed to stand overnight to permit some autolysis. After 0.2 g. calcium acetate had been added per 100 ml. of the extract, the temperature was raised to 70°C. for 15 min. The

liquid was cooled, clarified, and some thymol added. This extract was used as enzyme solution.

The hydrolysis products of a dry malt α-amylase preparation have already been described in chapter 1. Contamination of the enzyme by soluble, non-dialysable carbohydrate was shown to be 46%.

Action of salivary and pancreatic α-amylase on arabo-xylan: In a trial experiment using salivary α-amylase, the water extract and the enzyme were incubated at 37°C. for 24 hr. (a control containing a 0.2% starch solution with salivary α-amylase gave no colouration with iodine after this time). The mixture was boiled, evaporated to a small volume and clarified. Dialysis against running water was allowed to proceed for 3 days after which time the gum was precipitated with two volumes of acetone and slowly dried. The sugars galactose, glucose, arabinose and xylose were observed both in the amylase-treated product (P1) and in a parallel untreated product (P2). A further spot in the position of ribose was visible in the amylase-treated product; this sugar was known to be derived from the enzyme preparation.

In a similar trial experiment with pancreatic α-amylase, glucan content in the gum was found to be reduced but not eliminated, while galactan still remained.

Ammonium sulphate fractionation: It has been shown by Preece and Hobkirk (1953) that in the ammonium sulphate fractionation of the water-soluble cereal gums, galactan constituents remain in the mother-liquor. Also, since β-glucan is most effectively precipitated in the lower ammonium sulphate fractions, removal of these fractions, which contain at the same time some pentosan, might allow the precipitation of a pentosan free from β-glucan in the later, i.e. the 40-50% fraction.

Using the products P1 and P2, fractionation with ammonium sulphate was performed as follows: Each product was dissolved in water and ammonium sulphate was added gradually to a concentration of 30%. After stirring for 1 hr. and standing for 1 hr. in ice-cold water, the precipitate was removed and discarded. Ammonium sulphate was then added to the mother-liquor until its concentration was 50% and the resulting precipitate was removed by centrifugation, dissolved in water and the solution dialysed for 3 days. The gum was recovered by precipitation with 2 volumes of acetone. Chromatograms of the products (P3 and P4) showed that galactose and ribose had been eliminated but glucose still remained in both products, although in trace amount in the amylase-treated product P3. Plate I shows the products of hydrolysis of P1, P2 and P3.

Plate I.

Plate I

Chromatograms showing the products of acid hydrolysis of different samples of rye araboxylan (P1, P2 and P3)

P2 - crude acetone-precipitated rye araboxylan

P1 - product obtained by treating P2 with ∝-amylase

P3 - product obtained by ammonium sulphate fractionation of P1

K - control

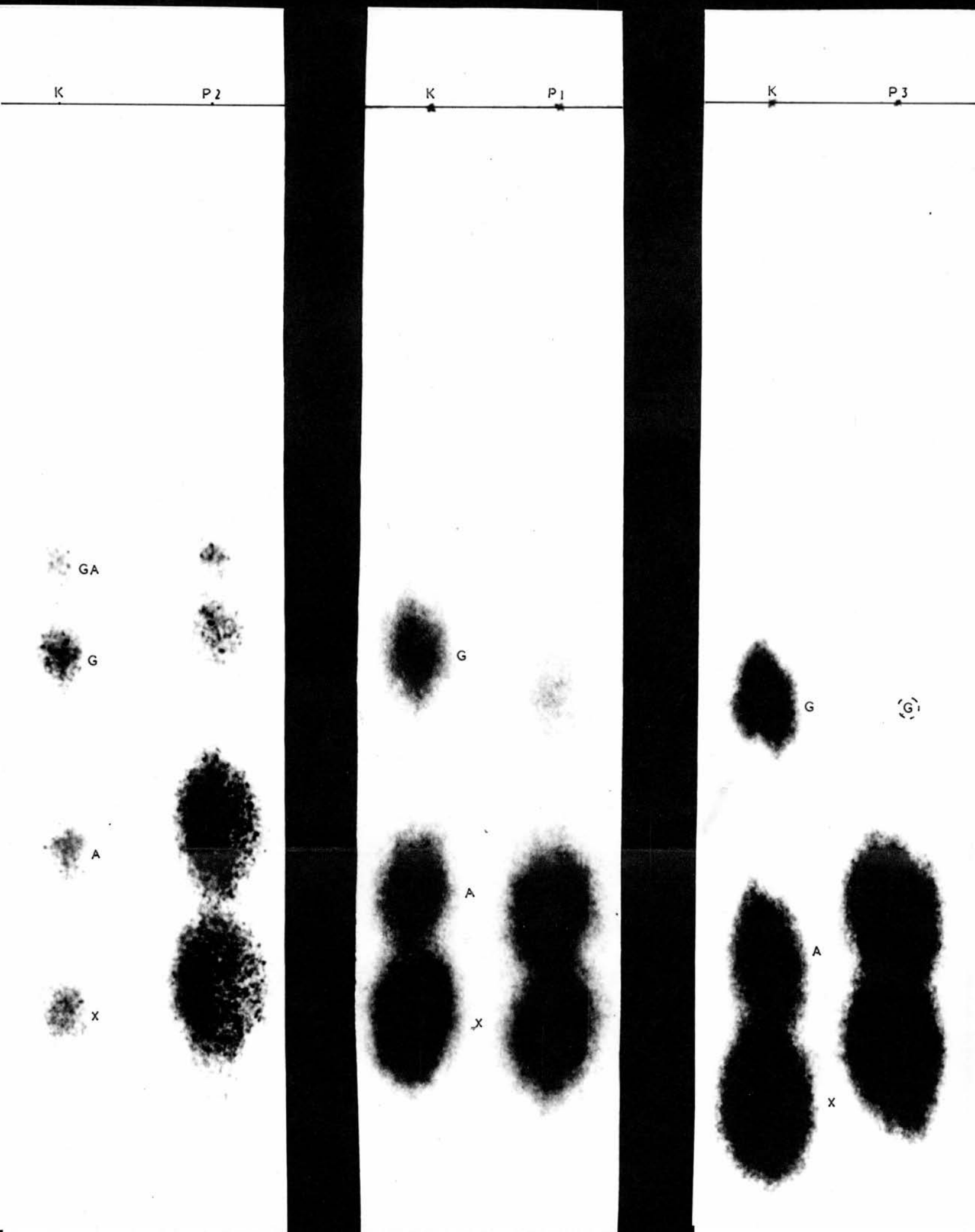
GA - galactose

G - glucose

A - arabinose

X - xylose

(G) - trace of glucose


K
P2
GA
G
A
X
K
P1
G
A
X
K
P3
G
(G)
A
X

Repeated amylase treatment: Since amylase treatment may have been inadequate, a further treatment with salivary α-amylase was performed on the product P3. Hydrolysis and chromatography of the product again indicated a trace of glucan; the conclusion seems unavoidable that this glucan is of β-linked nature.

Action of malt α-amylase on arabo-xylan: (i) 8 ml. of a 0.5% solution of araboxylan P11 (see Table I), 1 ml. of a citric acid - sodium hydrogen phosphate buffer of pH 3.5 and 2 ml. of a malt α-amylase extract were mixed and 10 ml. transferred to a viscometer. Over a period of 2 hr. at 25°C., the time of flow in the viscometer (water 18.4 sec.) changed by 2.5 sec. (93.6 to 91.1 sec.). This change was larger than desirable, but since glucose contamination in this substrate amounted to 6%, viscosity decrease may have been caused by enzyme action on glucan. (Glucan may have been present in the enzyme also). A chromatographic investigation of the low-molecular products of the reaction after 12 hr. at 25°C. showed a large amount of glucose and only a trace of arabinose, both of which might well have originated in the enzyme preparation. Arabinose production from the substrate would not be expected since the enzyme causing it is inactivated after heating at 60°C. for 10 min. (Preece and Hobkirk, 1955). The

action of a malt α-amylase extract on a β-glucan substrate was also investigated (Preece and Hoggan, 1957). A small, but negligible, decrease in viscosity was observed.

(ii) 0.48 g. arabo-xylan (P11) was dissolved in water and the volume made up to 100 ml. 20 ml. of the malt α-amylase extract were added and the mixture maintained at 65°C. for 2 hr. Boiling and concentration were followed by clarification and dialysis. The arabo-xylan was reprecipitated with 2 volumes of acetone. The yield was 0.46 g. (P14) and its composition is shown in Table I. The viscosity of the preparation was determined and it was found to have been reduced by about one fifth.

It is within the limits of experimental error to assume that the preparations P11 and P14 are of identical composition, (although the malt α-amylase extract may have contributed both to yield and to composition) the important feature being the unaltered glucose content. The decrease in viscosity, however, was not satisfactory since it appeared that some enzyme other than α-amylase was present causing shortening of the pentosan or β-glucan chains. No further attempt was made to use malt α-amylase in the preparation of pure arabo-xylan.

Chromatography and quantitative determination of sugars: Whatman No. 1 paper was used as inert support in qualitative experiments and Whatman No. 3MM in quantitative.

Descending chromatography with the upper layer of butanol-ethanol-water (45:5:50) as solvent and the lower layer as atmosphere saturant allowed the required separation of sugars in 4 days, although galactose and glucose have close R_F values in this solvent. For quantitative work, chromatography was used in conjunction with the Somogyi method. In order to ascertain the absence of galactose in gum hydrolysates, the "glucose" spots in several samples were eluted and run for 5-6 days in the above solvent; after this time, glucose and galactose in the controls had separated, and in all preparations where ammonium sulphate fractionation was employed, no galactose was detected.

Large-scale preparations of arabo-xylan: Preparations were made using 1,000 g. rye. Either salivary or pancreatic α-amylase was used in conjunction with two ammonium sulphate fractionations as described above. In several instances one fractionation was performed before and the second fractionation after amylase treatment while in other preparations both fractionations were performed after incubation with α-amylase.

A summary of the principal methods and the results obtained in the preparation of arabo-xylan is given in Table I.

TABLE I

Influence of various types of α-amylase on rye arabo-xylan

Preparation Number	Rye Sample	Pre-treatment	Amylase type	Post-treatment	Yield [(g)/1000g]	Ratio glucose:arabinose:xylose	Ratio arabinose:xylose
P2	I	Normal extraction	Nil	Acetone precipitation	5.0	ϕ13:31:56	36:64
P4	I	P2 as starting material	Nil	50%$(NH_4)_2SO_4$ precipitation*(1)	-	11:36:53	40:60
P7	I	50%$(NH_4)_2SO_4$* precipitation	Salivary	50%$(NH_4)_2SO_4$* precipitation	2.4	3:40:57	41:59
P8	II	Normal extraction	Nil	Acetone precipitation	-	ϕ23:29:48	38:62
P9	II	P8 as starting material	Salivary	50%$(NH_4)_2SO_4$*(a) precipitation	1.3	13:37:50	42:58
P10	II	as P9	Salivary	50%$(NH_4)_2SO_4$*(b) precipitation	1.4	5:37:58	39:61
P11	I	Acetone precipitation	Pancreatic	50%$(NH_4)_2SO_4$* precipitation	5.3	6:35:59	37:63
P12	I	Normal extraction	Pancreatic	30%$(NH_4)_2SO_4$ precipitation	3.2	7:35:58	38:62
P13	I	Normal extraction	Pancreatic	50%$(NH_4)_2SO_4$ precipitation ∠	2.1	3:39:58	40:60
P14	I	P11 as starting material	Malt	Acetone precipitation	-	6:37:57	39:61

*30%$(NH_4)_2SO_4$ fraction having been discarded

Mean 39:61

(a) immediate precipitate (b) delayed precipitate

(1) only one $(NH_4)_2SO_4$ fractionation here as compared with two in all other preparations

∠ 30%$(NH_4)_2SO_4$ fraction here is P12 ϕ glucose figure includes galactose

DISCUSSION

The use of the α-amylases in saliva and pancreatin in conjunction with ammonium sulphate fractionation has decreased considerably the amount of glucan contamination of the pentosan from rye. The obvious disadvantage in the use of the former enzyme source is the difficulty in obtaining sufficiently large quantities of the enzyme. Pancreatin is, therefore, preferable since standardisation of procedure is possible and action, after some purification, is equally, if not more, efficient. The use of malt α-amylase of necessity introduces the possibility of contamination of the pentosan by carbohydrate material present in the enzyme and it yields results, therefore, which are not altogether unequivocal.

The results indicate that α-amylase treatment fails to remove the residual glucan contamination of the rye pentosan prepared after preliminary α-amylase treatment and ammonium sulphate fractionation; we may reasonably conclude that this glucan is not of the 1:4- α-linked type but rather of the β-linked form. Amylase-treated products, unlike untreated preparations, gave no blue colouration with iodine. As is to be expected, the total amount of glucan present in the water-soluble rye gums varies from one sample to another. In sample I, on which most investigations were made, about 13% of the crude acetone-precipitated gum was of glucan or galactan

nature. About one quarter of this was β-glucan which could not be removed by ammonium sulphate fractionation; in other words, its molecular size was relatively small. The β-glucan content of the fraction precipitated by 30% ammonium sulphate was about 7%. In rye sample II, the initial amount of glucan present in the gum was larger than in sample I and the final glucan contamination was larger, indicating contribution by both α- and β-linked types.

An assortment of molecular sizes has often been postulated for β-glucan (Preece and Mackenzie, 1952b; Preece and Aitken, 1953; Preece and Hobkirk, 1953). Preece and Hobkirk (loc. cit.) assuming a direct proportionality to exist between viscosity and molecular size whether attributable to chain length or to molecular aggregation found that, as the precipitability of oat β-glucan with ammonium sulphate decreased, its molecular size also decreased. The same workers recorded from results of ammonium sulphate fractionation of rye, together with wheat and barley, gums that minimum glucan contamination occurred in the 40-50% salt fractions, those fractions precipitating at lower and higher salt concentrations being predominantly of β- and α-glucan nature respectively. In each of the three rye samples examined above, after α-amylase treatment, the 30% ammonium sulphate fraction

was found to contain a greater percentage of glucan than the 50%, a further suggestion of the β-linked nature of the glucan contamination.

No detailed study has been made of the nature of the glucan obtained from other cereals; Preece and Mackenzie (1952b) extracted gums from five common raw cereals precipitating them with Fehling's solution and acetone; by comparing the amounts of these precipitated in the 30% ammonium sulphate region, and also their viscosities and specific rotations, suggestions could be made concerning composition; it was later stressed, however, (Preece and Hobkirk, 1953) that precipitation with 30% ammonium sulphate was not a measure of β-glucan content. For example, barley and oats gave the highest total yields of gum, but whereas the barley product gave solutions of high viscosity and negative rotation, those from oats had low viscosity and positive rotation. Barley is known to be particularly rich in β-glucan which is precipitated by 20% ammonium sulphate and while the proportions of glucose, arabinose and xylose in the two products were not outstandingly different, the amount of gum precipitated from the barley product by 30% ammonium sulphate was twice that from oats. Preece and Hobkirk (loc. cit.) using ammonium sulphate fractionation directly on a cereal water extract suggested that glucan

in association with pentosan in the 40% oat fraction was of β-linked type; in this connection the earlier report of the presence of lichenin which is considered to be a linear polymer of β-D-glucose units containing 1:3- and 1:4-linkages in the proportion 3 to 7, in oats by Morris in 1942 is of interest. A study of the hemicellulosic materials present in barley during malting was made by Preece and Hoggan (1957), the water-soluble together with the alkali-soluble carbohydrates being examined for α- and β-glucan, as well as for pentosan, content. Malt α-amylase was used in the removal of α-linked glucan.

Preece and Mackenzie (loc. cit) referred to the low yield of gum from maize; also, while its composition and low viscosity bore similarities to the product from oats, its specific rotation was highly positive compared with the low positive rotation of the oats preparation. They suggested that the major part of the maize product whose low viscosity was in keeping with its low pentosan content was not essentially a typical gum but rather a type of water-soluble starch. The water-soluble glucan material from sweet corn has been investigated by Peat et al. (1956). They separated two products, depending on their differing solubilities in 67%

acetic acid, which were both shown to be "glycogen-type molecules of a polymer-homologous series". In β-amylolysis limit, iodine-stain, basal chain length and behaviour with R-enzyme (which has no action on either animal glycogen or phytoglycogen whereas it readily debranches amylopectin) the phytoglycogens, as they were named, were more closely related in structure to animal - or to yeast - glycogen than to amylopectin.

In another investigation of the composition of the acetone-precipitated gum-like polysaccharide from maize (Preece and Alexander, 1956), glucan, the major constituent, was found to be accompanied by small amounts of araban, xylan and galactan. Pancreatic α-amylase was used to remove dextrinous material; after dialysis, recovery by precipitation with acetone yielded a very small amount of gum-like material of which glucan, with galactan, were the minor constituents whereas araban exceeded xylan as the major constituents. Salivary α-amylase was used to confirm this result. This suggests that, of the 88% of glucan (together with galactan) which Preece and Mackenzie (loc. cit) reported present in the gum from maize (xylan content being 3% and araban 9%), less than 3% was of β-glucan type.

Perlin (1951) investigated the composition of

the soluble pentosans of wheat flours by extracting the flour with water at 15°C. and precipitating with alcohol. After acetylation of this crude material, fractionation with petroleum ether was performed on the acetate which had previously been extracted with acetone or chloroform. The various fractions were deacetylated and hydrolysed; glucose was the only constituent of the later fractions which in their reaction with iodine resembled starch or dextrin-like material. No suggestion was made as to the type of glucan (about 3%) present in the early pentosan-rich fractions. Wheat almost certainly resembles rye, therefore, in containing a small proportion of β-linked glucan as well as dextrinous glucan in its otherwise pentosan-rich gum.

In Table II, the compositions of the pentosan substrates used in the enzymic investigations reported in chapter 4, are summarised for reference and comparison. The relatively constant arabinose/xylose ratio is significant as is the failure to decrease glucan contamination below 3% of the gum. It has been found (chapter 4) that pure pentosan gum can be isolated after treatment of a glucan-contaminated gum with certain enzymes; such a procedure would not, however, be satisfactory if applied in the original preparation of the pentosan substrate since it is probable that some

TABLE II

Preparations of Rye Arabo-xylan*

Preparation Number	Rye Sample	$(NH_4)_2SO_4$ concentration (%) 1st precipitation	2nd precipitation	Yield [(g.)/1000g.]	Ratio glucose:arabinose:xylose	Ratio arabinose:xylose
Rc or P11	I	50	50	5.3	6:35:59	37:63
Re(i) or P12	I	30	30	3.2	7:35:58	38:62
Re(ii) or P13	I	50	50	2.1	3:39:58	40:60
Rf(i)	III	30	30	0.7	9:30:61	33:67
Rf(ii)	III	50	30	1.0	5:34:61	36:64
Rf(iii)	III	50	50	2.9	3:37:60	38:62
Rg(i)	III	30	30	2.1	7:31:62	33:67
Rg(ii)	III	30	50	0.8	7:34:59	37:63
Rg(iii)	III	50	50	1.6	3:43:54	44:56
Rh	IV	50	50	3.50	6:31:63	33:67
					Mean	37:63

* Pancreatic α-amylase and two $(NH_4)_2SO_4$ fractionations used in preparation.

modification, certainly removal of arabinose, would have resulted.

While grinding and abrasive treatment alone may be responsible for the production of soluble α- linked structures, their presence in the gums is nevertheless unavoidable; by enzymic degradation of these structures with α-amylase, however, there is every reason to suppose that they may be entirely eliminated and the glucan content of the water-soluble cereal gums separated into the two types of glucose-containing structure. In the preparation of pure pentosan from rye, the problem is obviously not confined to the removal of dextrinous material; β-glucan can largely be removed by 30% ammonium sulphate fractionation, but a small amount remains which can be precipitated only in the 40-50% region. Success in the removal of this residual glucan may now await the finding of specific inhibitors for pentosanases, thus allowing enzymic removal not only of α- but also of β-glucan.

SUMMARY

1. α- and β-linked glucans occur in all five common cereals, although the actual amounts of each type may vary, not only from one cereal to another, but also, although to

a lesser extent, amongst different samples of the one cereal.

2. Following α-amylase treatment to remove α-linked glucan, and ammonium sulphate fractionation to remove β-glucan and galactan, an araboxylan contaminated by only 3% glucan, presumably β-glucan, has been prepared from rye.

3. It is unlikely that these methods will allow the preparation of a pure pentosan from all rye samples, although some samples (as, for example, that used by Preece and Hobkirk in 1954) may be found which have a pronounced deficiency of β-glucan.

Chapter 3

PAPER ELECTROPHORESIS OF PENTO-OLIGOSACCHARIDES

INTRODUCTION

The technique of paper electrophoresis has in recent years developed simultaneously with the techniques of column and paper partition chromatography. Although paper electrophoresis is already extensively used as a clinical method for the separation of proteins, its potentialities in the carbohydrate field have been realised only comparatively recently. The most important step in the development of electrophoretic methods for carbohydrates was the use of borate buffers, introduced in 1952 by Consden and Stanier, by Michl, and, in the same year, by Jaenicke. The majority of the early investigations were concerned with monosaccharides, and some useful separations of these were achieved which had hitherto been very difficult by paper chromatography.

From a study of the electrophoretic behaviour of the mono-o-methyl-D-glucoses, it seemed likely that the reducing disaccharides of D-glucose with 1:2- or 1:4-linkages would have much smaller mobilities than those containing 1:3- or 1:6-linkages. This was confirmed experimentally by Foster (1953). It is apparently the linkage by which the "remainder" of the molecule is attached to the reducing moiety, rather than the structure of the "remainder", which determines mobility. An interesting application of this observation has been

in the examination of the products of enzyme action on the polysaccharide β-glucan (Hoggan, 1957). In addition to laminaritriose (containing two 1:3-β-linkages), a trisaccharide fraction, apparently pure by paper chromatography, was found to resolve, upon electrophoresis in borate buffer, into two components, whose chromatographic and electrophoretic mobilities could only be accounted for by the structures Glβ4Glβ3G and Glβ3Glβ4G.

The true mobilities of different molecules during electrophoresis cannot be determined directly, since there is always an electroendosmotic flow; a marker, such as 2, 3, 4, 6 tetra-o-methyl-D-glucose, which does not react with borate ions may, however, be used for reference. In this connection, it is important to note the observation of Bourne et al. (1956) and of Foster et al. (1956) that, during electrophoresis, different substances which do not complex with borate ions migrate at identical rates, under the influence of the electroendosmotic flow; thus there is little or no selective adsorption of low molecular weight carbohydrates.

Although carbohydrates having different molecular weights may have identical M_G values (M_G = true distance of migration of a substance/true distance of migration of D-glucose) when submitted to electrophoresis in

borate buffer, there are other buffers in which the molecular weight of an aldose determines its mobility. Thus Barker et al. (1956) have shown that the N-benzylglycosylamines of aldoses migrate as glycosyl-ammonium ions during electrophoresis in a formic acid-sodium formate buffer of pH 1.8; the mobilities are inversely proportional to the molecular weights of the ions, and also apparently independent of the actual structures of the sugars, and of the constituent linkages in di- and oligo-saccharides. Observations were made on a hexose series from the mono- to the hexa-saccharide level, and although the M_G values (represented by the mobility of the N-benzylglycosyl-ammonium ion, relative to that of N-benzyl-D-glucosylamine) for the higher oligosaccharides are perhaps too close to be unequivocal, those up to, at least, the tetrasaccharide level are satisfactory.

A similar method for the determination of molecular size of carbohydrates by electrophoresis was reported by Frahn and Mills (1956) who used a buffer of aqueous sodium bisulphite. An aldose apparently reacts slowly with bisulphite to form an anionic complex in equilibrium with the free sugar. During electrophoresis the complex migrates, but slowly decomposes during migration. Thus two spots may be observed, firstly that of the complex and secondly that due to electroendosmosis; the distance

between them is a measure of the true ionic mobility of the charged complex. The mobilities relative to glucose, for which M_G = 1.00, were found to be virtually identical for isomers and to decrease regularly, but not linearly, with increase in the molecular weight of the aldose.

No information was given by Barker et al. (loc. cit.) or by Frahn and Mills (loc. cit.) regarding the application of their methods to pento-oligosaccharides; some information in this direction is presented below.

EXPERIMENTAL

The apparatus used was that described by Foster in 1952.

Electrophoresis in the presence of borate.- Three pento-oligosaccharides which, on account of their chromatographic positions were probably di-, tri- and tetra-saccharides, were eluted according to the method of MacLeod (1951). The concentrates of the eluates were analysed electrophoretically on Whatman No. 1 paper by the method of Foster (1953) in which the electrolyte is 0.2 M sodium borate of pH 10.0 (Clark and Lubs). Plate I shows the result of electrophoresis at a potential gradient of about

Plate I.

Plate I.

Paper electrophoresis of pento-oligosaccharides as borate complexes at 20 volts/cm. and 5-7 milliamps for 3 hr.

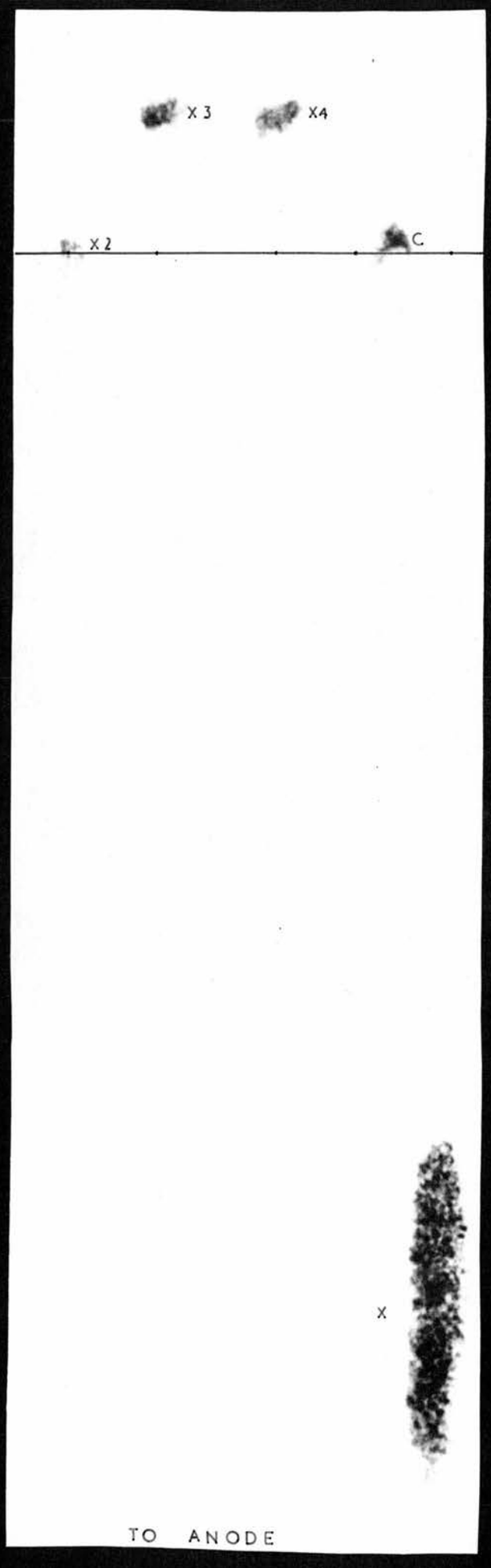
X - xylose

C - cellobiose

X2 - xylobiose

X3 - pento-trisaccharide, probably xylotriose

X4 - pento-tetrasaccharide


X3
X4
X2
C
X
TO ANODE

20 volts/cm. and 5-7 milli-amps. for 3 hr. The spots were revealed by spraying the paper with aniline oxalate acidified with glacial acetic acid.

Electrophoresis in the presence of formic acid-sodium formate.- The electrolyte Barker et al. (loc. cit.) consisted of a mixture of 5% aqueous sodium hydroxide solution (600 c.c.) and 90% formic acid (400 c.c.). Glucose, xylose and oligosaccharides which from their chromatographic positions and acid hydrolyses were probably xylobiose, xylotriose and a pento-tetrasaccharide mixture were dissolved in a solution containing 1 c.c. of benzylamine in 9 c.c. methanol and 5 c.c. N formic acid. After spotting on Whatman No. 1 paper (9 cm. wide and 56 cm. long) and immediately before electrophoresis, the paper was heated at 95°C. for 5 min. Plate II shows the result of electrophoresis at 650-700 volts and 45 milli-amps. for 6 hr. The spots were revealed by dipping the paper in an acetonic solution of silver nitrate, and thereafter spraying with an alcoholic solution of sodium hydroxide. The distance separating the complexed from the uncomplexed sugar was calculated; M_x values (M_x = the distance separating the two spots for a particular sugar/the distance separating the two spots for xylose) were calculated and are shown in Table I.

Electrophoresis in the presence of bisulphite.- The

Plate II.

Plate II (i)

Paper electrophoresis of pento-oligosaccharides in the presence of formic acid-sodium formate.

Plate II (ii)

Paper electrophoresis of pento-oligosaccharides in the presence of bisulphite.

G - glucose

X - xylose

X2 - xylobiose

X3 - xylotriose

X4 - pento-tetrasaccharide mixture

G^{1}, X^{1}, $X2^{1}$, $X3^{1}$, $X4^{1}$ represent the N-benzylglycosylamines of the corresponding aldoses.

G^{11}, X^{11}, $X2^{11}$, $X3^{11}$, $X4^{11}$ represent the bisulphite complexes of the corresponding aldoses.

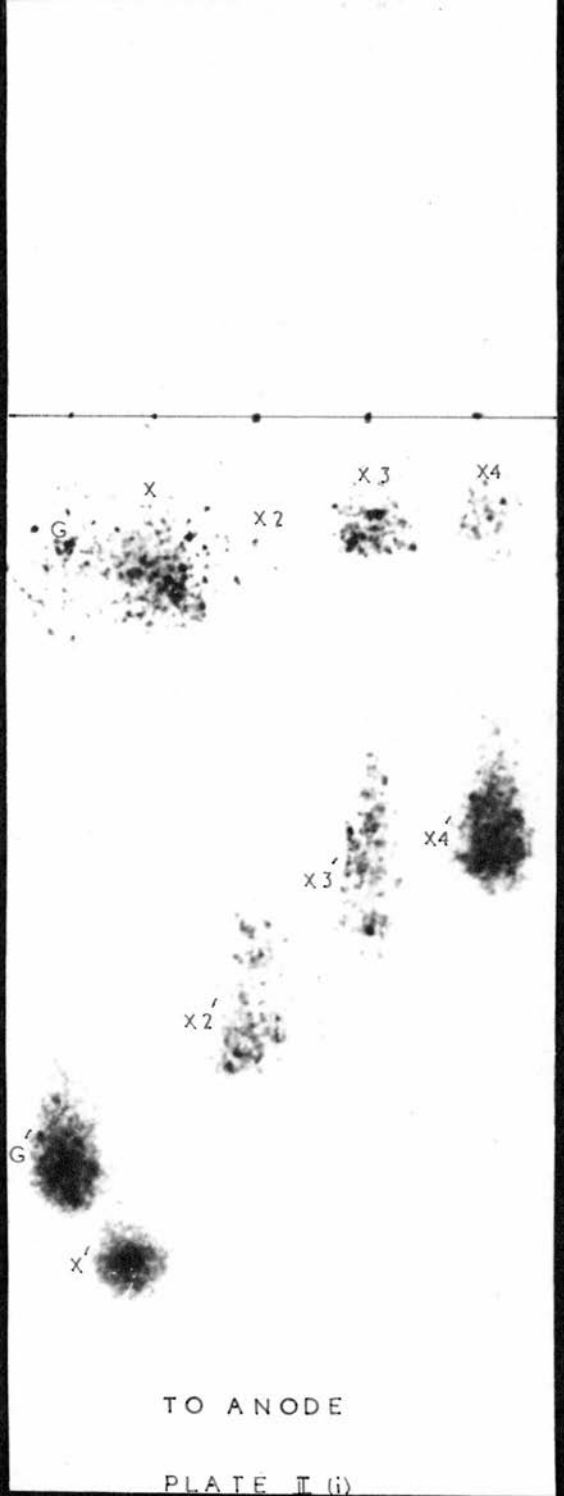


PLATE II (i)

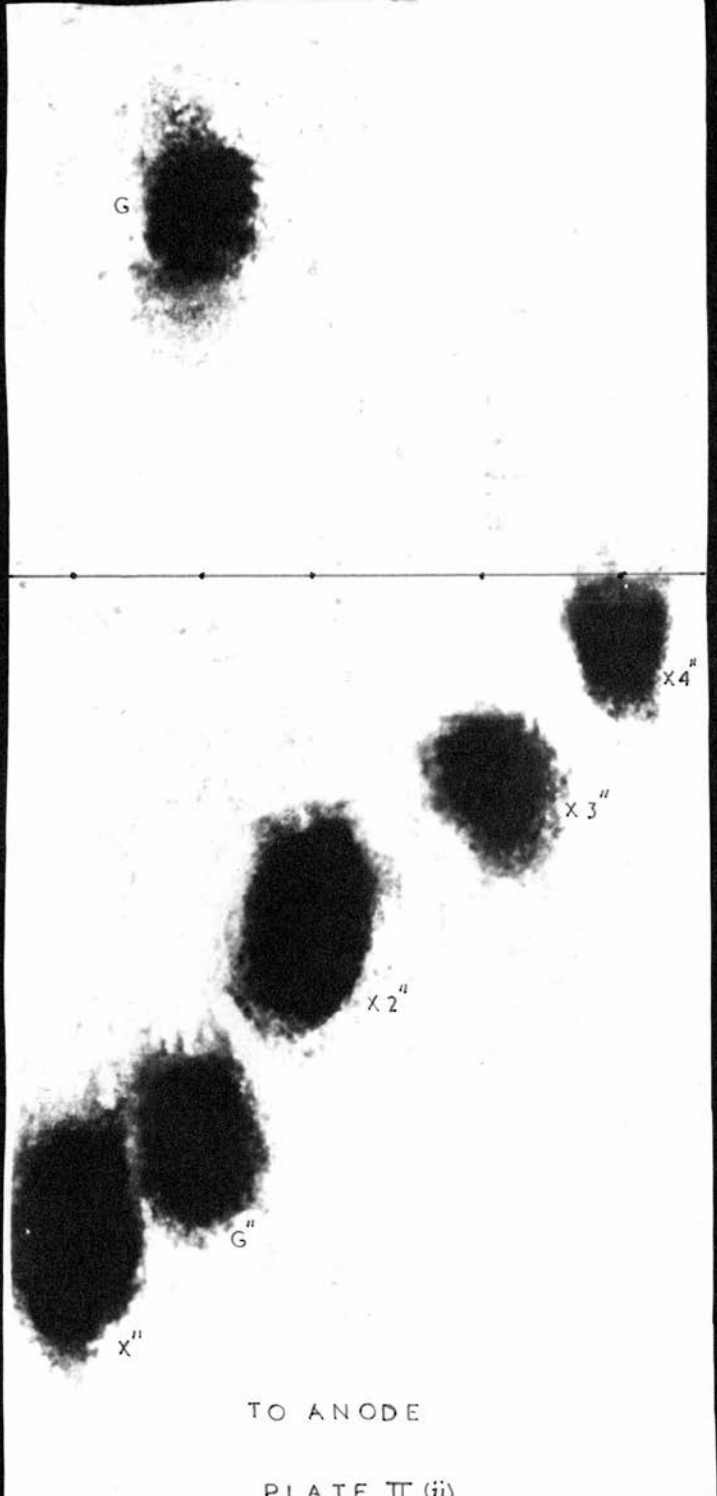


PLATE II (ii)

TABLE I

Electrophoresis of Pento-oligosaccharides in the Presence of Formic acid-sodium formate.

	Mx*	1/Mx	Molecular Weight
Xylose	1.00	1.00	150
Xylobiose	0.74	1.35	282
Xylotriose	0.59	1.70	414
Pento-tetrasaccharide	0.46	2.17	546

* Mx = Mobility of pento-oligosaccharide relative to that of xylose.

method was essentially that of Frahn and Mills (loc. cit.), but, on account of the small quantities of the sugars available, their solutions in 0.4 M sodium metabisulphite, after being allowed to stand for ½ hr. to permit complex formation, were spotted on the dry paper and not on paper equilibrated in the electrolyte. By repeated application of the solution, sufficient sugar was present for electrophoresis. Glucose, xylose and what were probably xylobiose, xylotriose and a pento-tetrasaccharide mixture were subjected to electrophoresis in 0.4 M sodium metabisulphite at 500-600 volts and 50 milli-amps. for 5-6 hr.; Whatman No. 4 paper, 8 cm. wide and 50 cm. long was used. The spots were revealed by dipping the paper in a solution of aniline picrate in acetone, followed by heating at 100°C. A typical electrophoretogram is shown in Plate II and the results are recorded in Table II. An attempt to increase the applied voltage by decreasing the concentration of the sodium metabisulphite by half resulted in less discrete spots. Apparently there is almost 100% complex formation between pentose sugars and bisulphite, and the electroendosmosed spots of the uncomplexed sugar after electrophoresis are not evident; for this reason, the corresponding glucose spot was utilised as reference point. An error was thus introduced since it was observed that cellobiose did not always possess the same electroendosmotic mobility as glucose,

TABLE II

Electrophoresis of Pento-oligosaccharides in the presence of sodium metabisulphite.

	M_G * range	Average M_G	$1/M_G$	Molecular Weight	Calculated M_G ✓
Glucose	1.00	1.00	1.00	180	
Xylose	1.15-1.18	1.16	0.86	150	1.14
Xylobiose	0.73-0.78	0.76	1.31	282	0.77
Xylotriose	0.57-0.60	0.58	1.72	414	0.58
Pento-tetrasaccharide	0.45-0.51	0.47	2.13	546	0.47

* M_G = mobility of pento-oligosaccharide relative to that of glucose

✓ M_G calculated from the equation M_G (M.W. + 129) = 317

although the results of Bourne *et al*. (1956) and of Foster *et al*. (1956) infer that, at least in borate buffer, non-complexing substances migrate at identical rates due to electroendosmosis.

DISCUSSION

Electrophoresis in the presence of borate.- Foster (1953) correlated the mobilities in borate buffer of certain glucodisaccharides and of methyl derivatives of glucose with their stereochemistry. Complex formation was suggested to be based mainly on three types of structure: (A) *cis*-hydroxyl groups at $C_{(2)}$:$C_{(4)}$, (B) *cis*-hydroxyl groups at $C_{(1)}$:$C_{(2)}$, (C) hydroxyl groups at $C_{(4)}$:$C_{(6)}$. If, in a methyl derivative of glucose or in the reducing moiety of a glucodisaccharide, (A) and (B) were possible, M_G values would be high; if (B) or (C) alone were possible, M_G values would be moderate; if (A), (B) and (C) were impossible, M_G values would be low. It was found that if (A) were precluded, there was a considerable fall in the M_G value, suggesting (A) to be of major importance in determining mobility.

Applying this theory to pento-oligosaccharides, we observe that xylobiose, xylotriose and higher xylo-oligosaccharides, with only (B) possible, would be

expected to possess low M_G values. Since the pento-oligosaccharides were derived from rye pentosan, the presence of the disaccharide arabinosyl-3-o-β-D-xylose might also be expected on the basis of structural investigations by Aspinall and Sturgeon (1957); it should have a fairly high mobility, since (A) would be possible as well as (B). The mixed trisaccharides represented by $\overset{a}{x}$-x (the reducing end being assumed at the right) or by x-$\overset{a}{x}$ should possess little mobility. This is in agreement with the experimental results. There is no evidence for the presence of the mixed disaccharide which should be easily distinguishable, by its greater mobility, from xylobiose. Apparently, molecular weight is reflected to some degree in mobility, for xylobiose has greater mobility towards the anode than has the tri- or tetra-saccharide.

Electrophoresis in the presence of formic acid-sodium formate.- A regular, but not linear, fall in the M_x values (Table I) is observed when these are plotted (Fig. 1) against the molecular weights of the corresponding pento-oligosaccharides. When the reciprocals of the M_x values, however, are plotted against molecular weights, the points for the mono-, di- and tri-saccharides are collinear and that for the supposed tetrasaccharide lies close to this line. Certainly its position results in little doubt as to the size of the molecule.

Figure 1.

Figure 1.

Electrophoresis of pento-oligosaccharides in the presence of formic acid - sodium formate.

a. Molecular weight plotted against M_x (the mobility of the pento-oligosaccharide relative to that of xylose).

b. Molecular weight plotted against $^1/M_x$.

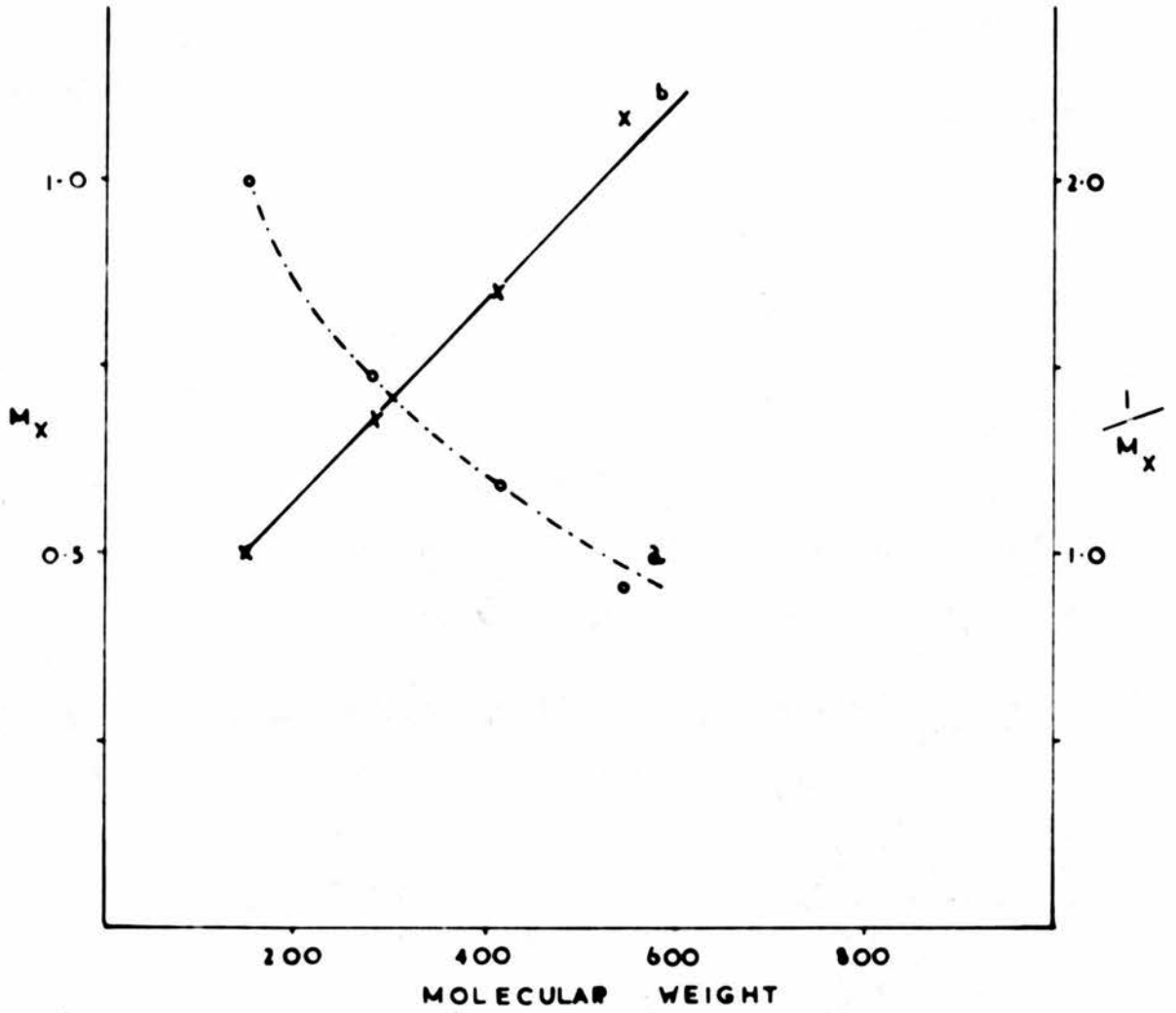


FIG. 1

Electrophoresis in the presence of bisulphite.- From the experimental values given by Frahn and Mills (loc. cit.) the equation $(M_G+7)(M.W.+232.5) = 48{,}665$ was calculated by Hoggan (1957), where M_G is the mobility of the hexo-oligosaccharide, relative to glucose, and M.W. is its molecular weight. A straight-line relationship was found to exist when the reciprocal of M_G was plotted against molecular weight.

The M_G values and their reciprocals for the pento-oligosaccharides are plotted against molecular weights in Fig. 2; a linear relationship is seen to exist between the reciprocal of M_G and the molecular weight of the oligosaccharide. While the inadequacy of the available results is realised, considerable agreement is found to exist (Table II) between the observed M_G values and those calculated from the equation M_G (M.W.+129) = 317.

The application of the techniques already available for the determination of molecular size of oligosaccharides of the hexose series, will provide similar information for pento-oligosaccharides. Thus, although chromatography alone fails to yield reliable evidence for molecular weights, on account of the dependence of mobilities upon constitution, it will, in conjunction with electrophoresis, furnish such information when only micro-quantities of the oligosaccharides are available.

Figure 2.

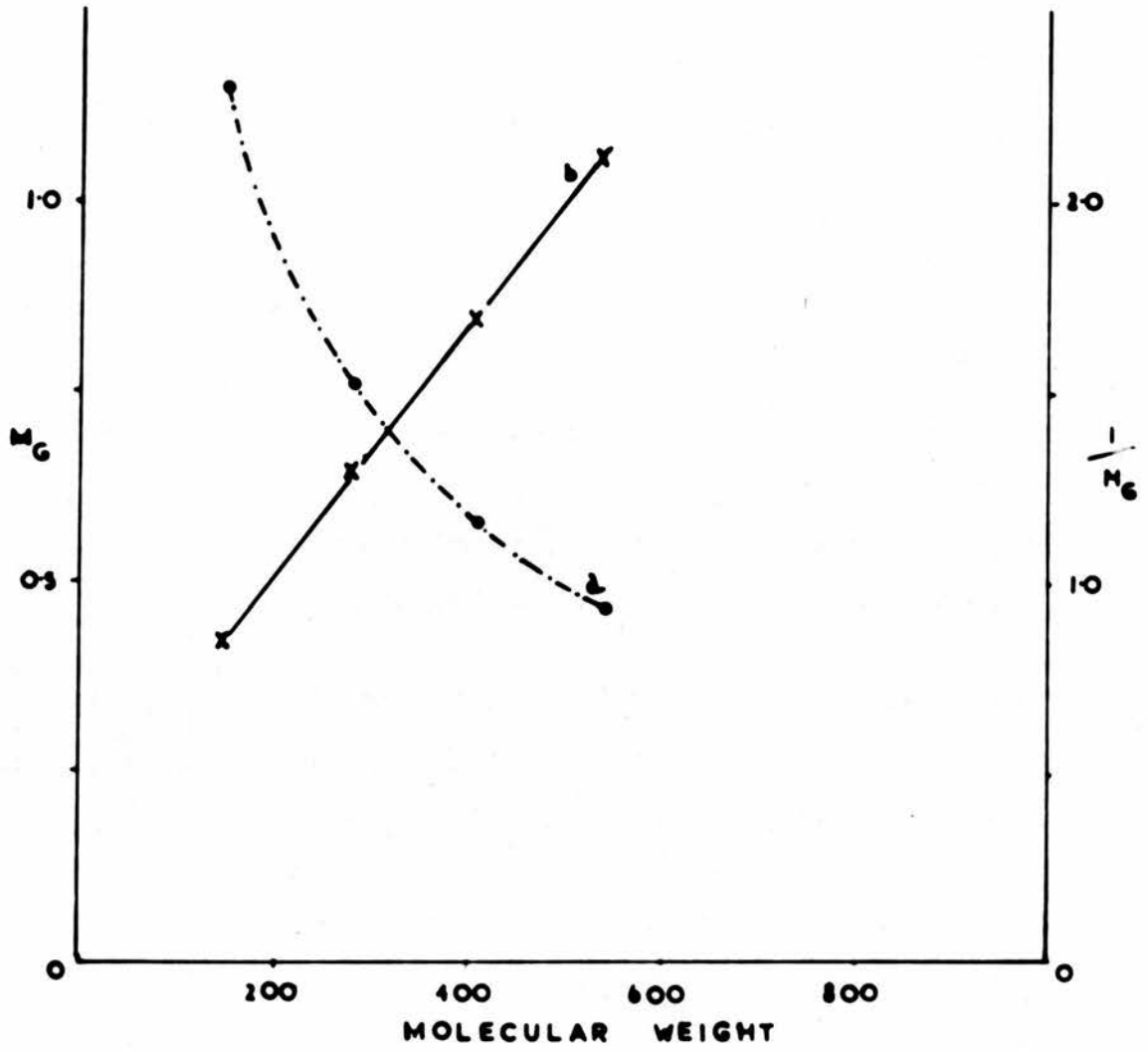


FIG. 2

Figure 2.

Electrophoresis of penta-oligosaccharides in the presence of sodium metabisulphite.

a. Molecular weight plotted against M_G (the mobility of the penta-oligosaccharide relative to that of glucose).

b. Molecular weight plotted against $^1/M_G$.

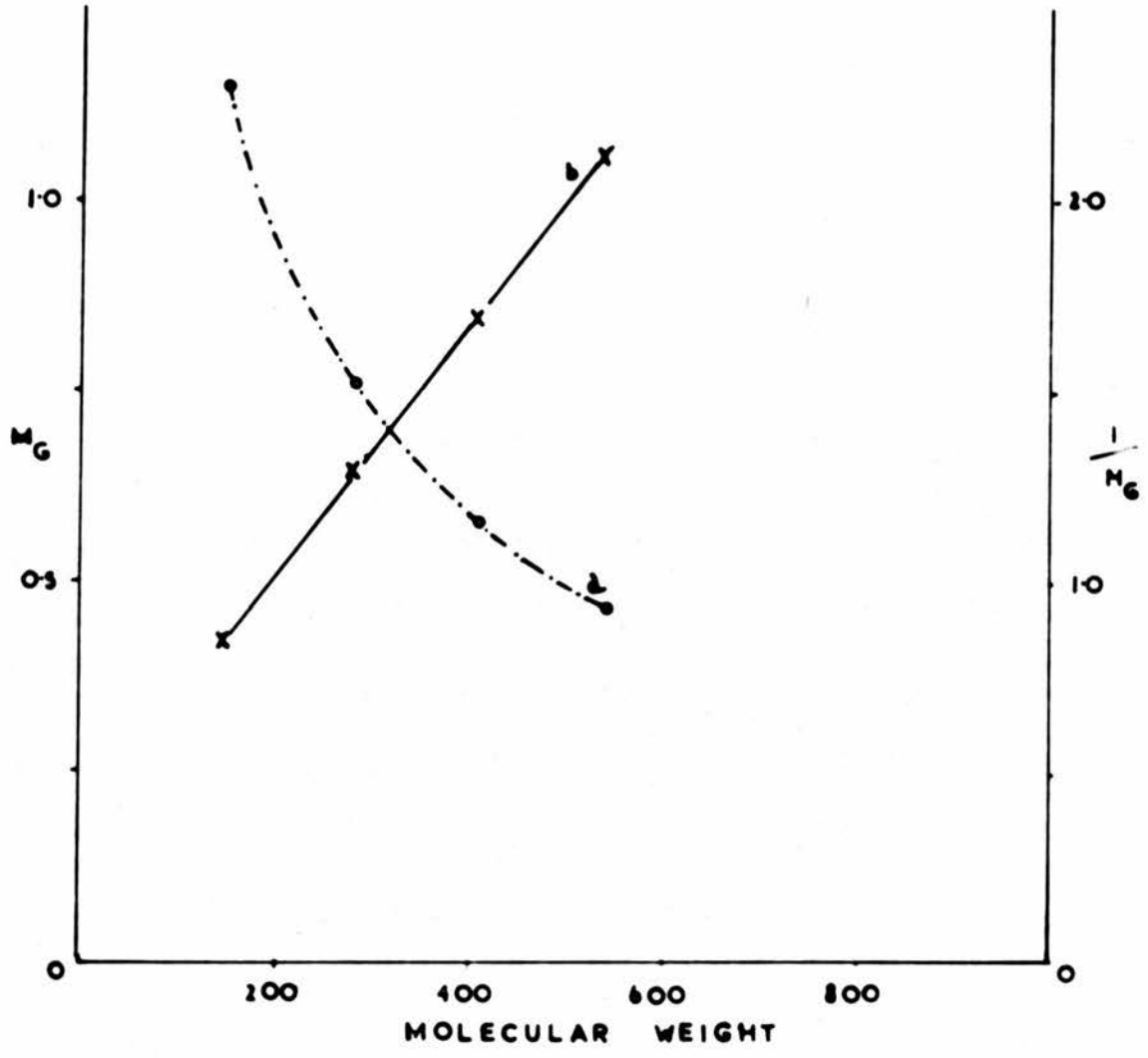


FIG. 2

SUMMARY

1. The electrophoretic behaviour of pento-oligosaccharides from the di- to the tetra-saccharide level in the presence of borate buffer has been investigated; the mobilities have been found to be in agreement with the theory of Foster (1953) which related to glucodisaccharides and methyl derivatives of glucose.

2. The electrophoretic methods for the determination of molecular size of hexose-oligosaccharides, reported by Barker et al. (1956) and by Frahn and Mills (1956), have been extended to pento-oligosaccharides.

Chapter 4

THE ENZYMOLYSIS OF RYE ARABOXYLAN

INTRODUCTION

Since 1952, when Preece and Mackenzie evolved a technique for the fractionation of the water-soluble, non-starchy polysaccharides of cereal grains, there has been an accumulation of information regarding both the structural features of β-glucan and its degradation by cereal enzymes; although the pattern of chemical structure of the pentosans is already generally acknowledged, little information is yet available concerning the cereal pentosanases. The salient difficulty in an investigation of the pentosanase characters of cereals lies in the low activities of the enzymes concerned, a feature observed 30 years ago by Lüers and Volkamer (1928) when, using an enzyme preparation from green malt, they achieved some hydrolysis of xylans from barley grain and elder pith. The enzymic decomposition of xylan originating from plum pits by an enzyme from barley malt was investigated by Voss and Butter (1938a) who investigated the kinetics of the reaction and demonstrated the production of xylose. The production of pento-oligosaccharides, but not of free pentoses was observed by Bass et al. (1953) during the action of raw barley enzymes on mixed barley gum; enzymes from malt, however, yielded free pentoses. In a preliminary investigation of the enzymolysis of a

comparatively pure pentosan from rye, by a barley enzyme, Preece and Hobkirk (1955) demonstrated a rapid initial production of free arabinose, and a slow production of pento-oligosaccharides.

A brief survey of the literature concerning investigations of pentosan degradation by enzymes from sources other than cereals has already been given in the General Introduction. Perhaps the most informative account is that of Sørensen (1957); he used chromatography and increase in reducing power to determine the reaction products and activities, respectively, when enzymes liberated from soil microorganisms acted upon colloidal wheat-straw xylan. Hydrolysis of xylan by extracellular enzymes was found to proceed in two stages. During the initial period, there was a rapid production of reducing groups with the formation of oligosaccharides containing from two to six or more xylose residues, in addition to mixed oligosaccharides, containing arabinose or uronic acid, as well as xylose. In the second stage, increase in reducing power was slow, requiring incubation for several days, when the end-products were xylobiose, xylose, arabinose and uronic acid. A random attack on the xylan chain was postulated.

The production of mixed arabinose-xylose oligosaccharides from wheat-straw xylan by an enzyme from Myrothecium verrucaria was previously reported by

Bishop and Whitaker (1955), whose results confirmed the existence of arabinose units as components of the xylan molecule. The production of mixed pento-oligosaccharides from water-soluble cereal pentosan has not as yet been reported, although evidence for the co-existence of arabinose and xylose units in wheat pentosan (Perlin, 1951; Montgomery and Smith, 1955) and in rye pentosan (Aspinall and Sturgeon, 1957) has been provided by methylation data.

While chromatography is a useful guide in a qualitative examination of enzyme action, a clearer conception of the nature of the enzymic attack can be obtained only from quantitative experiments. In the case of a water-soluble cereal gum, diminution in the viscosity of its solution in the presence of the enzyme serves as an indication of endo-activity. Extreme care must, however, be used in interpreting such results, if the substrate is not pure. Comparison of the endo-β-glucanase activities reported by Preece and Hoggan (1956) and the endo-xylanase activity of an enzyme preparation from barley recorded by Preece and Hobkirk (1955) shows the former system to be many times more active on β-glucan than the latter on pentosan. Thus the results of Simpson (1954), who determined the pentosanase activity of certain fungi, streptomycetes and bacteria by the viscosity drop of a solution of wheat "pentosan", are questionable; only 80% of the substrate was pentosan

which may, therefore, have been accompanied by as much as 20% of glucan, whose viscosity would also have been decreased in the presence of a suitable enzyme.

A second method widely employed in the determination of enzymic activity is the measurement of reducing-group liberation (Preece and Hobkirk, loc. cit.; Sørensen, loc. cit.). Again, the use of a pure substrate is essential if reliable information is to be obtained. It must be remembered that, in such investigations, the number of reducing groups may be increased by the action of more than one enzyme system, and that no more that a comprehensive pattern of enzymic activity may be deduced.

In the present investigation, namely of the enzymolysis of rye araboxylan by cereal enzymes, the quantitative method adopted yields unambiguous results concerning the production of arabinose; viscosity diminution of the substrate solution is used in a general assessment of endo-activities, which are considered comparatively rather than absolutely; the production of free xylose is studied quantitatively. Chromatographic and electrophoretic methods are applied in the investigation of the products of enzymic action.

EXPERIMENTAL

I Viscometric investigations

Before using the viscometric method for comparison of the endo-xylanase activities of various enzyme preparations, it was necessary to establish the validity of the method, in view of the β-glucan contamination of the pentosan substrate (see chapter 2).

Comparison of the endo-xylanase and endo-β-glucanase activities of enzyme preparations obtained from grain during the commercial malting of Proctor barley. The enzymes were those whose preparation was described by Preece and Hoggan (1956); viscometric determinations were carried out under the conditions specified by Preece and Aitken (1953). Rye araboxylan (P11) in 0.5% solution and the enzymes in 0.5% solution were used with a phosphate-citrate buffer (Britton, 1942) of pH 3.5. The results for endo-β-glucanase activities (Preece and Hoggan, 1956) and for endo-xylanase activities are recorded in Table I, and are plotted against the time of processing of the grain in Fig. 1.

Validity of viscometric determinations for endo-xylanase activity. In view of the enormous activity increase in the β-glucanase system, it might be assumed that the very small amount of β-glucan in the araboxylan would be destroyed in the first few minutes of action of the enzyme

TABLE I

Endo-β-glucanase and Endo-xylanase Activities of Enzyme Preparations from Grain during the Commercial Malting of Proctor Barley.

Stage	Time (hr.)	Endo-β-glucanase $\not\emptyset$	Endo-xylanase $\not$
Raw Barley	-	0.007	0.0007
Steep 1	-	-	-
2	65	0.011	0.0009
3	89	0.009	0.0007
Floor 1	24	0.010	0.0007
2	48	0.006	0.0008
3	72	0.013	0.0015
4	96	0.035	0.0022
5	168	0.077	0.0032
Kiln 1	12	0.133	0.0026
2	22	0.074	0.0016
3	42	0.092	0.0018

$\not$ Increase in reciprocal specific viscosity per hr. per mg. enzyme; units are arbitrary and based on standard substrate.

$\emptyset$ The results quoted are those of Preece and Hoggan (1956), the preparations having been made available by them for pentosanase determination.

Figure 1.

Figure 1.

Changes in the endo-β-glucanase and endo-xylanase activities of enzyme preparations obtained from grain during the commercial malting of Proctor barley.

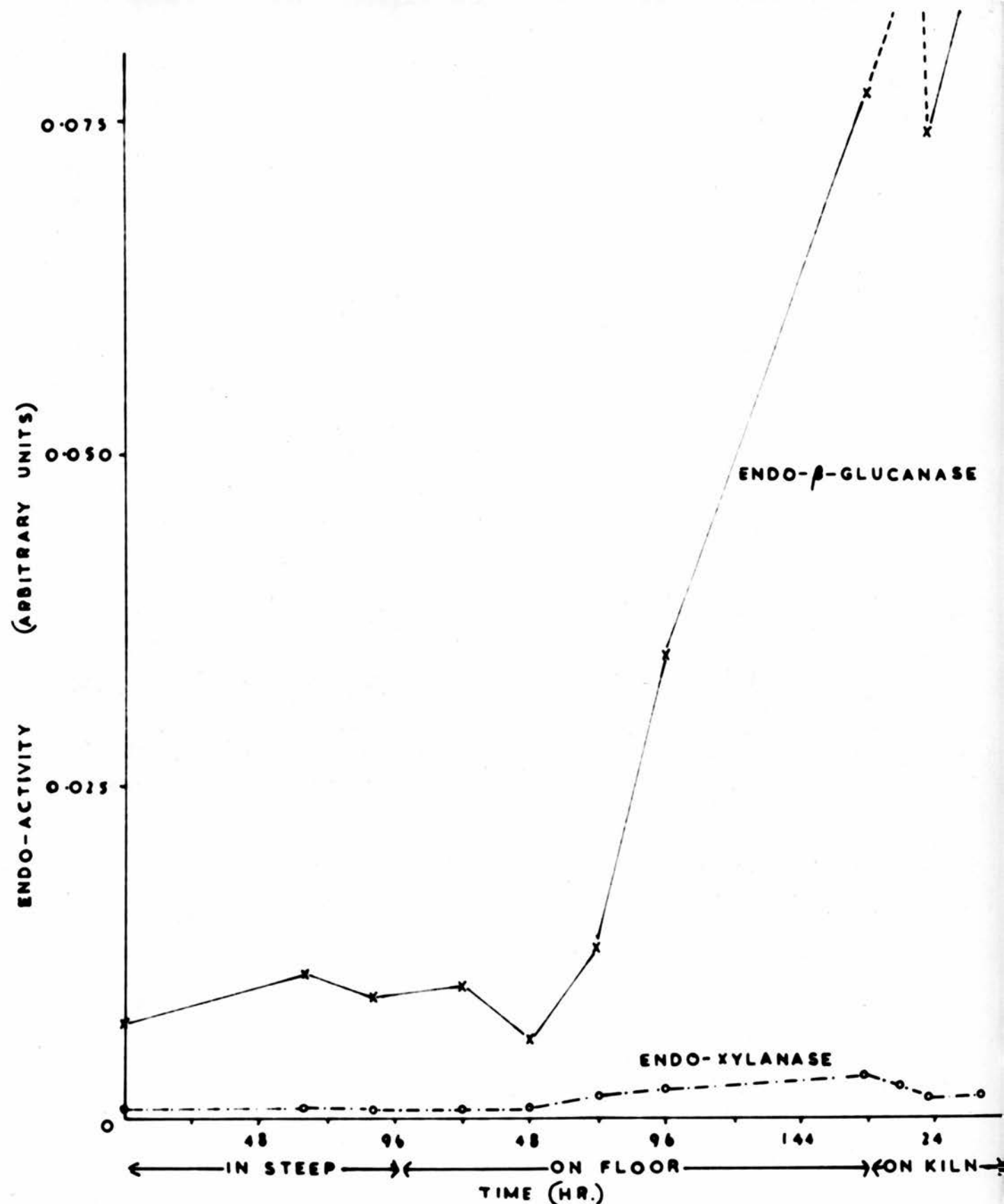

0·075
0·050
0·025
0
ENDO-ACTIVITY (ARBITRARY UNITS)
ENDO-β-GLUCANASE
ENDO-XYLANASE
48
96
48
96
144
24
IN STEEP
ON FLOOR
ON KILN
TIME (HR.)

FIG. 1

preparations, at least in the later stages of malting. Thus, 10 mg. of an enzyme preparation with an endo-β-glucanase activity of 0.100 units/mg./hr. would reduce the specific viscosity of a preparation of β-glucan, with initial specific viscosity of 4, to about 2 in 15 mins., and to 1 in 30 mins., under the conditions used and with an initial 55 mg. of β-glucan. There is, in fact, no more than 3-4 mg. of β-glucan present in 55 mg. of the araboxylan preparation, and although, by the Michaelis principle, this will not be eliminated proportionately more quickly, it is inevitable that, in the presence of such active endo-β-glucanases, the small amount of β-glucan is unlikely to remain in a viscous form for many minutes. Certainly, as will be shown later, the rapid degradation of the β-glucan contamination is manifested in the absence of all hexosic material, other than glucose itself, after a 6 hr. incubation of a 'pentosan' substrate with a barley enzyme.

There are two other factors to be considered; precipitation at a concentration of 40% ammonium sulphate yields β-glucan of low viscosity and the specific viscosity of this contaminant is unlikely to exceed 4, if even to approach it, whilst subsequent change in viscosity when the specific viscosity is below 1 is extremely slow, even in the presence of an active enzyme.

The conclusion cannot be escaped that the influence of the degradation of 6% of β-glucan upon pentosanase activity, as measured by viscosity drop, is small; this conclusion is strengthened by the failure to observe any departure from linearity in the reciprocal specific viscosity/time graphs, even in the earliest stages of action of enzyme preparations known to be rich in endo-β-glucanase. Three conclusions follow: (a) Endo-xylanase activity can be measured (at least as a first approximation) by viscosity drop, in the presence of minor amounts of β-glucan. (b) Endo-xylanase activity is distinct from endo-β-glucanase activity since no parallelism of enzyme action is evident in Fig. 1 for the two substrates. (c) Whereas preparations of very great endo-β-glucanase activity (here up to 20 times that of preparations from raw barley) can be obtained from malt at appropriate stages of modification, the malting enhancement of pentosanase activity in these preparations is much smaller (up to 5 times).

Endo-xylanase activities of enzyme preparations from common cereals. The enzymes were prepared from 100 g. of cereal according to the method described in Chapter 1. Two samples of each cereal were examined; in Series I, the enzymes were precipitated with acetone and taken to dryness over a period of 2 days; in Series II, the precipitates with

acetone were extracted with water, the insoluble material discarded, and the enzymes reprecipitated with acetone; they were dried in acetone over a period of 3 hr. The yields of the enzyme preparations are comparable within the respective Series, and are shown in Table II. The enzymes were stored in a refrigerator.

Endo-xylanase activities were determined as described above, using the enzyme preparations in 0.2% aqueous solution. The results are shown in Table II.

II Quantitative liberation of arabinose and xylose. General method. 0.1850 g. araboxylan was dissolved in 25 ml. water and 22.5 ml. of this solution was incubated at 37°C., with 1.67 ml. phosphate-citrate buffer (Britton, 1942) of pH 5.0 and 7.5 ml. of a 0.2% enzyme solution. Thymol was added as antiseptic. 5 ml. aliquots were withdrawn after 6, 16, 25, 40 and 50 hr.; they were heated in boiling water for 3 min., cooled and 1 ml. of ribose solution, containing 500 µg. ribose, was added, followed by 12 ml. ethanol. After shaking, the mixture was stored in a refrigerator for at least 6 hr. The precipitated gum was removed by centrifugation, the centrifugate filtered through kieselguhr, and evaporated to dryness. The mixture was dissolved in a small volume of 30% ethanol and streaked on Whatman No. 3 MM chromatographic paper (7½ in. x 3½ in.); the chromatogram

TABLE II

Endo-xylanase Activities of Enzyme Preparations from Common Cereals.

Cereal	Series I *		Series II *	
	Yield ø	Endo-xylanase ⌿	Yield ø	Endo-xylanase ⌿
Barley	0.41	0.008	0.34	0.013
Maize	1.00	0.009	0.09	0.018
Oats	0.36	0.010	0.38	0.027
Rye	1.67	0.007	0.34	0.012
Wheat	1.27	0.004	0.62	0.007

* Series I and II relate to different samples of the cereals.

ø Yield in g. per 100 g. of cereal.

⌿ Increase in reciprocal specific viscosity per hr. per mg. enzyme; units are arbitrary and based on standard substrate.

Substrates in Series I and II were P7 and Rh respectively.

was irrigated by the ascending method with n-propanol-water (78:22) for about 8 hr. In this manner, low-molecular material was separated from residual protein and gum, which cause retardation and poor separation during descending chromatography. The sugars were eluted (MacLeod, 1951) and spotted on Whatman No. 1 paper; two spots of the solution were applied in the central portion of the chromatogram, with a control spot at each side. Descending chromatography in the upper layer of the solvent butanol-ethanol-water (45:5:50) effected the separation of arabinose, xylose and ribose in 5 to 6 days. The portion of the chromatogram corresponding to one of the test spots was cut out and the remainder of the chromatogram was sprayed with aniline oxalate solution. The positions of the pentoses were marked off and, after examination of the two control strips, allowance was made, if necessary, when the solvent front had not run parallel to the starting line. The appropriate portions of the paper were cut off and the pentoses eluted. Each eluate was transferred to a Pyrex test-tube (15 cm. long and 1.5 cm. in diameter) and made up to 2 ml. with distilled water. Determinations were carried out according to the colorimetric method of Fernell and King (1953). The straight-line relationship between the amount of pentose and the colorimeter reading is identical for

arabinose, xylose and ribose. The amounts of arabinose and xylose corresponding to 500 µg. ribose, and hence to the original 5 ml. aliquot taken, were calculated.

Recovery experiments using the above method were satisfactory, 90-95% recovery of pentoses being obtained.

Application of the quantitative method to enzyme preparations from common cereals. The above method was applied as follows: (a) Enzyme preparations from the five common cereals (Series II) were used. The substrate for the maize enzyme was Rh and for the others Rg (iii). The results are shown in Table III. (b) Substrates Rf (iii), Rg (iii) and Rh were each incubated with the barley enzyme. The residual gums, precipitated after incubation of the enzyme for 6 hr. and 25 hr. with substrate Rf (iii), were hydrolysed and their compositions determined. No glucose was found in the hydrolysates and the arabinose/xylose ratios were 38/62 and 36/64 for the residual gums from the 6 and 25 hr. incubations respectively, while in the original substrate the ratio was 38/62. (c) Substrate Rg (iii) at half the standard concentration was incubated with the barley enzyme. (d) A pentosan 'dextrin' was prepared by precipitation of the residual gum, after incubating substrate Rf (iii) with the barley enzyme for 25 hr. The yield was 0.1600g. 'dextrin' from 0.3700 g. Rf (iii); the 'dextrin' contained arabinose and xylose in the ratio 36/64. An

TABLE III

Quantitative Liberation of Arabinose and Xylose by Enzymes from Common Cereals.

Cereal	Weight of pentose (/u.g.) liberated at given time intervals[*]														
	6 hr.			16 hr.			25 hr.			40 hr.			50 hr.		
	A	X	X_2	A	X	X_2	A	X	X_2	A	X	X_2	A	X	X_2
Barley	241	-	±	590	40	+	848	98	++	1260	295	++[Ø]	972	527	+++[Ø]
Maize[/]	157	-	-	310	-	-	434	37	±	866	66	+	915	165	+
Oats	223	±	-	515	±	+	886	50	++	1059	367	++[Ø]	1774	604	++[Ø]
Rye	243	-	-	635	26	+	897	46	+	1090	228	+	983	217	+
Wheat	151	-	-	465	-	-	355	27	-	796	134	-	1090	250	+

[*] Weight of pentose (/ug.) in 5 ml. aliquot.

[Ø] Pento-oligosaccharides higher than X_2 also produced.

[/] Substrate Rh; Rg (iii) for other cereals.

Symbols A, X, X_2 denote arabinose, xylose and xylobiose respectively.

incubation mixture was prepared using 0.1480 g. 'dextrin' as substrate (with appropriate volumes of buffer and enzyme solution) for the barley enzyme, and 5 ml. aliquots were removed after 6, 16, 25 and 40 hr.

The results of (b), (c) and (d) are recorded in Table IV.

Substrate controls (in which the enzyme solution was replaced by distilled water) and enzyme controls (in which the substrate solution was replaced by distilled water) were similarly incubated. No mono- or oligo-saccharides, other than ribose, were detected after incubation for 50 hr. at 37°C.

Plate I shows a typical series of chromatographic strips for the 5 incubation periods with the barley enzyme.

Test for enzymic synthesis: A mixture of arabinose and xylose, in concentrations similar to those found in the above mixtures after about 40 hr. incubation, was incubated with barley enzyme for 50 hr. at 37°C. No evidence was found for enzymic synthesis, either upon chromatographic examination or quantitative determination. It must be emphasised that, by an alteration in the conditions, enzymic synthesis might occur, but certainly it would appear that, in the above experiments, synthetic mechanisms are unlikely.

TABLE IV

Quantitative Liberation of Arabinose and Xylose using Different Samples of Araboxylan with the Barley Enzyme.

Substrate		Weight of pentose (µg.) liberated at given time intervals														
		6 hr.			16 hr.			25 hr.			40 hr.			50 hr.		
Prep. No.	Ratio A:X	A	X	X_2	A	X	X_2	A	X	X_2	A	X	X_2	A	X	X_2
Rf (iii)	38:62	260	±	±	750	114	+	1001	236	++	1459	559	++			
Rg (iii)	44:56	241	-	±	590	40	+	848	98	++	1260	295	++	972	527	+++
Rh	33:67	306	-	-	721	±	+	928	217	144	1052	500	256	1016	647	382
25 hr. 'dextrin'	36:64	247	38	38	587	93	87	584	364	192	969	607	375			
Rg (iii)[*]	44:56	237	-	-	631	53	+	629	161	+	738	286	++	1110	390	++

[*] Concentration of substrate $\frac{1}{2}$ standard concentration.

Symbols as in Table III

X_2 figures are in terms of xylose equivalents.

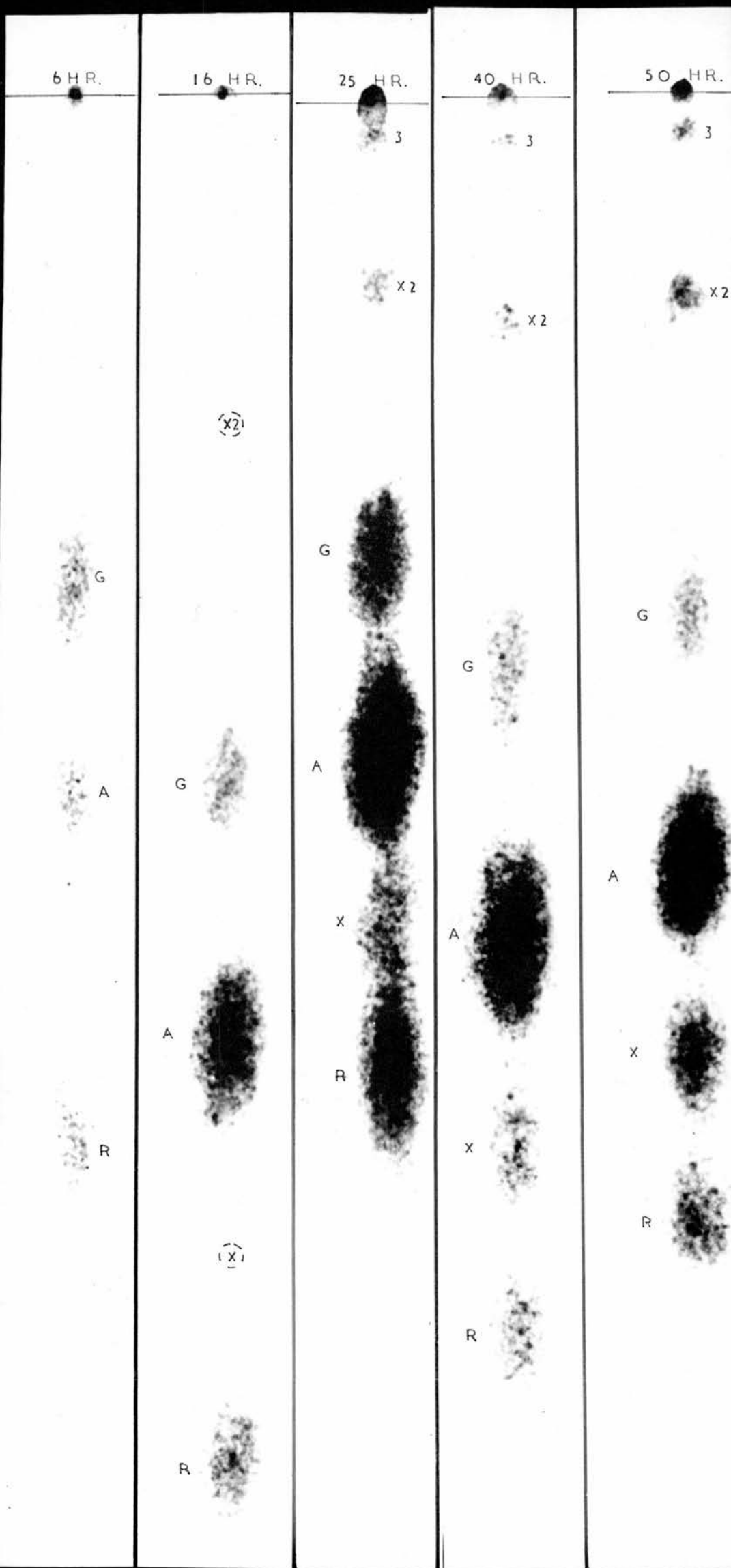
Plate I.

Plate I.

Chromatograms showing the products of enzymic hydrolysis when rye araboxylan was incubated for the periods shown with an enzyme preparation from barley (Solvent: upper layer of butanol-ethanol-water, 45:5:50). For quantitative purposes, a standard amount of ribose was added to each 5 ml. aliquot withdrawn from the incubation mixture.

R - ribose
X - xylose
A - arabinose
G - glucose
X2 - xylobiose
3 - pento-trisaccharide mixture

(X), (X2) represent trace quantities of the corresponding pentoses.


6 HR.
16 HR.
25 HR.
40 HR.
50 HR.
3
X2
(X2)
G
A
R
X
(X)

III Chromatographic investigations

Enzyme preparations from oats were used in these investigations. Mixtures for incubation were similar to those described in the General method of part II. Incubations were made at both 25°C. and 37°C. for 48 hr. with no noticeable difference in the products. Thymol was added to each mixture as antiseptic. After the residual gum had been removed as described above, the mixture was evaporated to dryness.

Separation of oligosaccharides. Two methods were applied and found to be equally satisfactory. (i) Paper chromatography: The residue was taken up in 30% ethanol and streaked on chromatograms of Whatman No. 3 MM paper which were then irrigated by the ascending method using n-propanol-water (78:22) for about 18 hr. A lengthwise strip was sprayed with aniline oxalate solution and a series of sections of the chromatogram above the monosaccharide level were eluted and evaporated. Fresh chromatograms were prepared with these mixtures, together with a control, usually raffinose; they were irrigated as above, the time of irrigation depending upon the complexity of the oligosaccharide fraction. Thus di-, tri- and tetra-saccharide fractions were obtained; higher oligosaccharides, including some which remained on the starting line, could not be separated from each other. (ii) Charcoal column: Charcoal and celite were mixed in

the proportions 2 to 3 by weight. Using distilled water, a slurry was made and this was added in small amounts to the column which contained a plug of glass wool covered by a layer of celite. The column was washed with water until the pH of the eluate was that of distilled water. An aqueous solution of the products of enzyme action was added to the column which was then washed with water (monosaccharides and some disaccharide removed) and then with 20% aqueous ethanol which removed oligosaccharides. Separation of the oligosaccharides was performed either as above in (i), or by the application of descending chromatography in the solvent (upper layer) butanol-acetic acid-water (40:10:50). Separation was improved by multiple development, the chromatogram being dried every second day and re-irrigated; 7 to 8 days were required to separate the tetrasaccharide from higher oligosaccharide fractions.

Acid hydrolysis of an oligosaccharide: The oligosaccharide was eluted from the chromatogram and hydrolysed in a sealed tube with 0.1N hydrochloric acid for 4 hr. at 100°C. The hydrolysate was taken to dryness at 40°C. on a vacuum hotplate, beside solid sodium hydroxide. The products were separated chromatographically on Whatman No. 1 paper using the upper layer of butanol-ethanol-water (45:5:50). The use of N hydrochloric acid for hydrolysis, or drying at 100°C., caused reversion of the products to xylobiose, etc.

Results of acid hydrolysis of oligosaccharides:

(i) Disaccharide fraction: All samples of this fraction examined contained only xylose, and it was concluded that this oligosaccharide was xylobiose. This was confirmed by its behaviour during paper electrophoresis (see Chapter 3). When the substrate P12 was used, samples of xylobiose were contaminated with a hexosic disaccharide of R_F slightly less than that of xylobiose (see plate V); upon hydrolysis, this anomalous material appeared to yield glucose and mannose.

(ii) Trisaccharide fraction: Using the descending method of chromatography, only one spot was obtained for this fraction, which sometimes yielded only xylose on hydrolysis, but which more frequently yielded both arabinose and xylose. When the ascending method in n-propanol-water was applied, this fraction separated into 2 components, one which yielded arabinose and xylose in the ratio 0.83:2.0 (a high xylose figure is expected on account of acidic degradation of arabinose) and the other, whose mobility in the above solvent was less, which yielded only xylose on hydrolysis. Paper electrophoretic studies were in agreement with the assumed trisaccharide nature of the xylose-containing compound, but the amounts of the mixed compound available were insufficient to give a reliable picture of electrophoretic behaviour. It was concluded that the compounds were an arabinosyl-xylosyl-

xylose and xylotriose.

(iii) Tetrasaccharide fraction: Again, only one spot was visible when the descending method of chromatography in butanol-acetic acid-water was applied; upon hydrolysis, the fraction occasionally yielded only xylose, but, more often, both xylose and arabinose. The ascending chromatographic procedure showed that the fraction was not homogeneous, but failed to permit clear separation of the components. It is certain that xylotetraose is sometimes, if not always present (the molecular size of a sample containing only xylose was confirmed by paper electrophoresis); the identity of the other component (or components) is still under investigation; it is almost certain that an arabinosyl-xylosyl-xylosyl-xylose is present; there may, however, be a tetrasaccharide containing 2 arabinose and 2 xylose units, or a pentasaccharide containing arabinose and xylose in the ratio 2/3.

(iv) Pentasaccharide and higher oligosaccharide fractions: Plate II shows the result of hydrolysing 1 cm. portions from the starting line to what was probably the pentasaccharide level of a chromatogram run in butanol-acetic acid-water for 8 days using multiple development. Each portion contained arabinose and xylose and it is probable that each represents a mixture of oligosaccharides. It is clear that the amount of oligosaccharide as well as the

Plate II.

Plate II.

Chromatogram showing the result of hydrolysing (with 0.1N hydrochloric acid) 1 cm. portions from the starting line to what was probably the pentasaccharide level of a chromatogram run in butanol-acetic acid-water for 8 days using multiple development. (Solvent: Butanol-ethanol-water, 45:5:50).

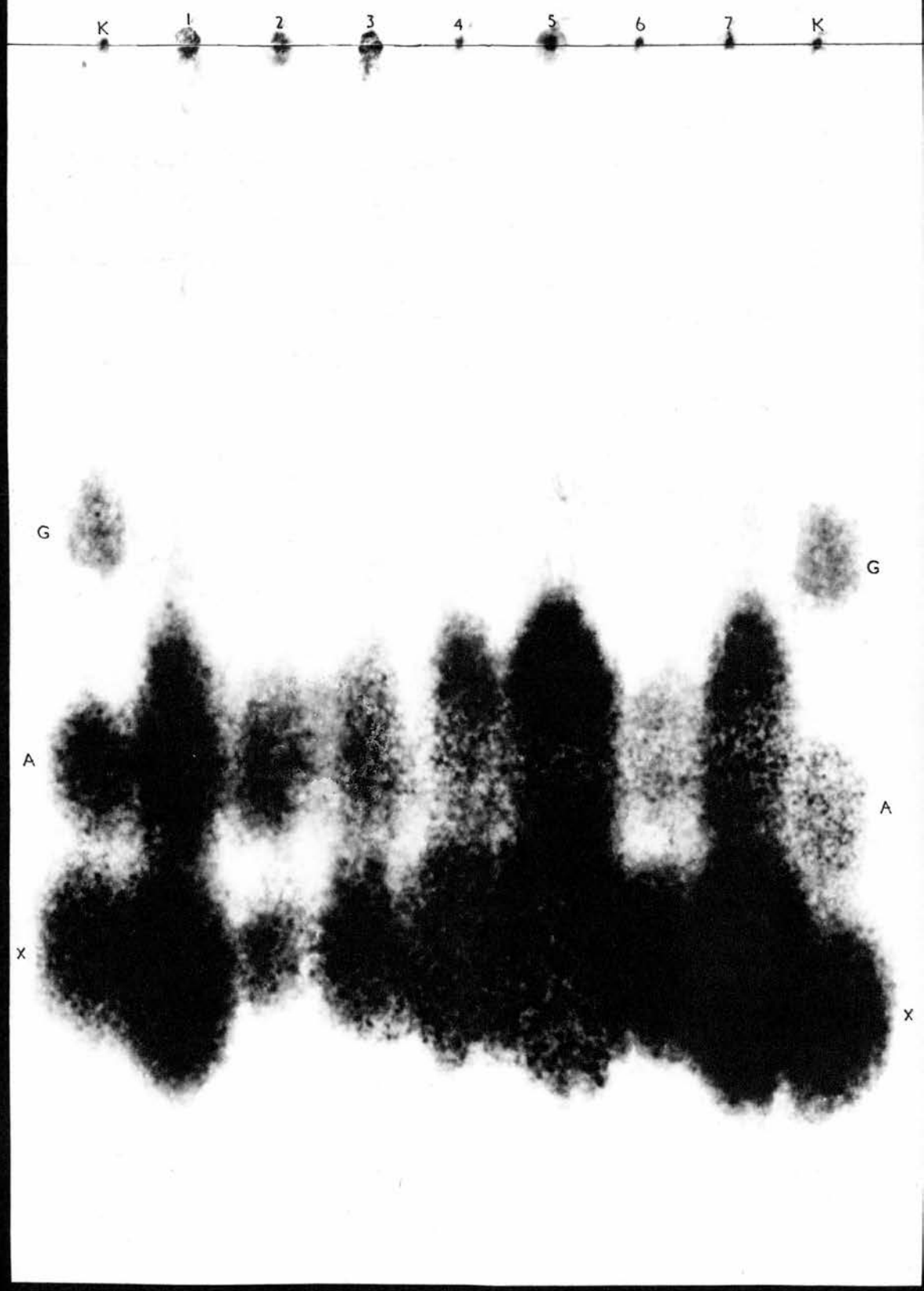
The hydrolysis products of the 1 cm. portions are numbered 1-7, number 1 being at the starting line of the original chromatogram and number 7 probably at the pentasaccharide level.

K - control

G - glucose

A - arabinose

X - xylose


K
1
2
3
4
5
6
7
K
G
A
X
G
A
X

arabinose/xylose ratio varies from one portion to another. Acetic acid hydrolysis (N acetic acid at 100°C. for 4 hr. in a sealed tube) of similar portions of a chromatogram containing mixed oligosaccharides, yielded an apparently homologous series of xylo-oligosaccharides (see plate III) from xylotriose to xylohexaose; as mixed oligosaccharides, therefore, levels higher than the hexasaccharide must be present, the hydrolysis with acetic acid having removed the arabinosyl residues.

Multiple development using the ascending method also fails to give sufficient separation to allow of unequivocal results when the arabinose/xylose ratios, after hydrolysis of the oligosaccharide with hydrochloric acid, are determined.

Plate IV shows the chromatographic separations achieved when the 20% aqueous ethanolic eluate from a charcoal column is run for 1, 2, 3 and 4 days in n-propanol-water and for 6 days in butanol-acetic acid-water. The speed and greater efficiency of separation using the former method recommend its use.

R_F values of pento-oligosaccharides: (a) Using the ascending method with n-propanol-water (78:22), average values of R_G values (R_F glucose = 0.31) of xylobiose, arabinosyl-xylosyl-xylose and xylotriose were 0.50, 0.35 and 0.26 respectively. (b) Using the descending

Plate III.

Plate III.

Chromatogram showing an apparently homologous series of xylo-oligosaccharides from xylose to xylohexaose obtained by acetic acid hydrolysis of 3 portions of a chromatogram containing mixed arabinose-xylose oligosaccharides. (Solvent: butanol-acetic acid-water, 40:10:50).

K - control

L, M, N - hydrolysis products of different portions of the original chromatogram

G - glucose

A - arabinose

X - xylose

X2 - xylobiose

X3 - xylotriose

X4 - xylotetraose

X5 - xylopentaose

X6 - xylohexaose

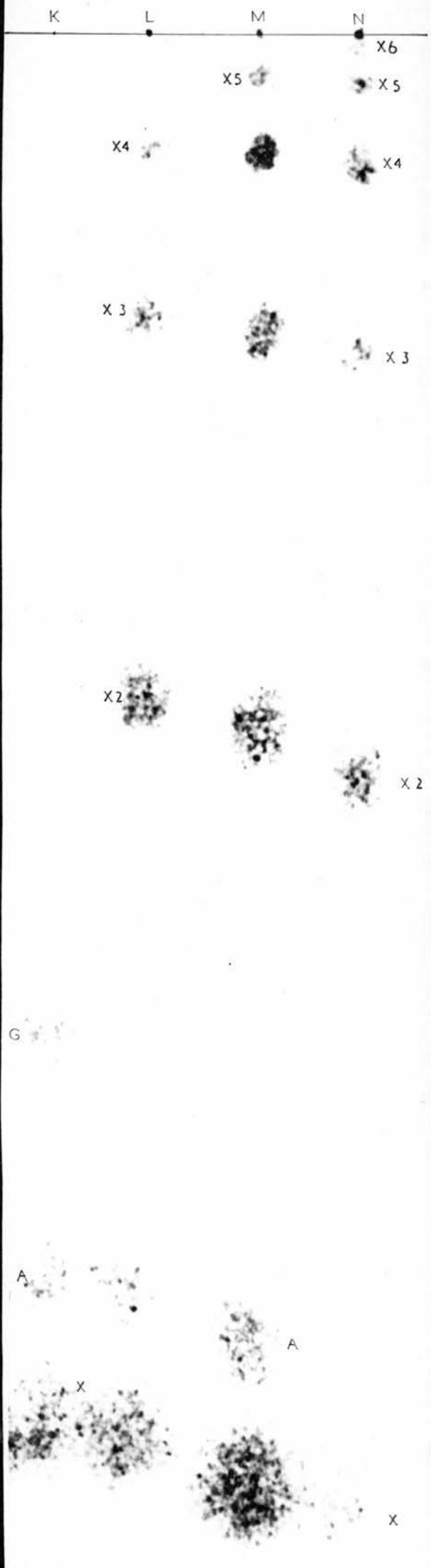

K
L
M
N
X6
X5
X5
X4
X4
X3
X3
X2
X2
G
A
A
X
X

Plate IV.

Plate IV.

Chromatograms showing the separations achieved when a 20% aqueous ethanolic eluate from a charcoal column (which had been used in the preliminary separation of the products of enzymic hydrolysis when rye araboxylan had been incubated for 48 hr. at 37°C. with an enzyme preparation from oats) is run for 1, 2, 3 and 4 days in n-propanol-water (78:22) and for 6 days in the upper layer of butanol-acetic acid-water (40:10:50).

L - chromatogram run in n-propanol-water for 1 day

M - " " " " 2 days

N - " " " " 3 days

P - " " " " 4 days

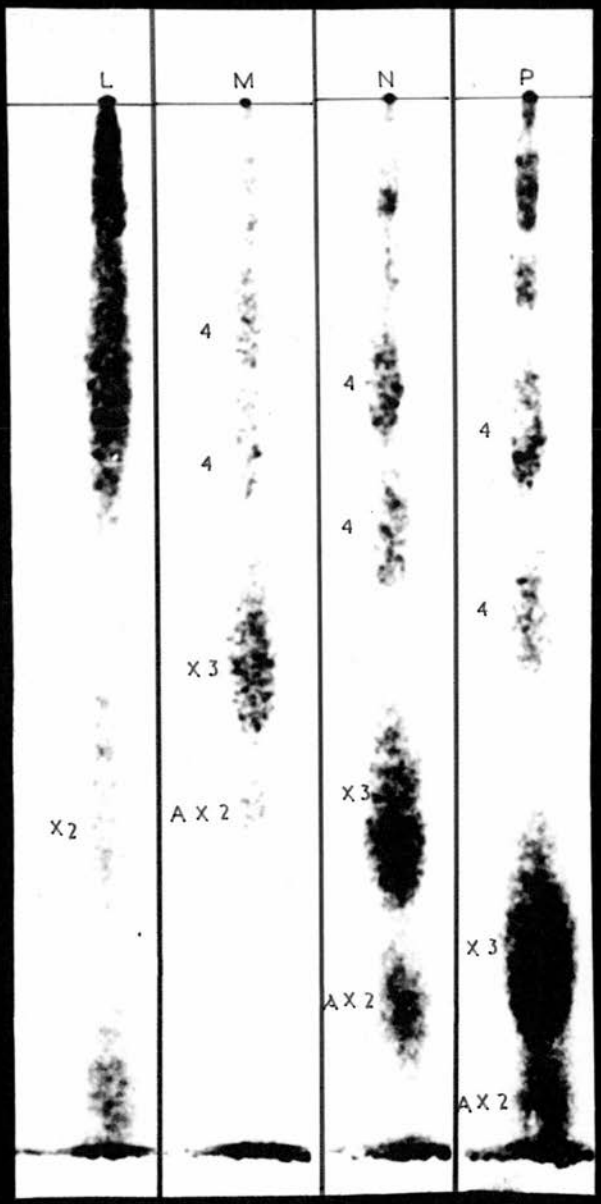
Q - " " in butanol-acetic acid-water for 6 days

X2 - xylobiose

X3 - xylotriose

AX2 - arabinosyl-xylosyl-xylose

4 - pento-tetrasaccharide fraction


L
M
N
P
Q
4
4
4
4
4
4
4
X3
X3
X3
X2
A X 2
A X 2
A X 2
X 3,
A X 2
X 2

method with butanol-acetic acid-water, R_F values varied widely and only those from the same chromatogram could be compared.

Action of an enzyme preparation from oats upon xylobiose and xylotriose: Xylobiose and xylotriose were each eluted from a chromatogram, and incubated with an enzyme preparation from oats for 24 hr. at 37°C. Chromatographic examination of the products (see plate V) confirmed the presence of a xylobiase and of an enzyme which splits xylotriose into xylose and xylobiose. Controls were run in parallel for enzymes and substrates; they were in all cases satisfactory.

Differential inactivation: It has been shown (Preece and Hoggan, 1956) that differential inactivation of the enzymes responsible for β-glucan degradation i.e. endo-β-glucanase, exo-β-glucanase and cellobiase, may be achieved by the use of various chemical agents. On the basis of the experiments of Preece and Hoggan (loc. cit.) the effects of the treatment of an enzyme preparation from oats with calcium acetate, phenylacetic acid and phenylmercuric nitrate, under the conditions shown in Table V were investigated. In each case, 40 mg. of the oats enzyme preparation in 0.2% solution was used. Following the prescribed treatment, each solution was dialysed for 3 days against running water, the enzyme

Plate V.

Plate V.

Chromatograms showing the products when xylobiose and xylotriose are each incubated with an enzyme preparation from oats for 24 hr. at 37°C.

(Solvent: butanol-acetic acid-water, 40:10:50).

K - control

E.K. - enzyme control

L - products when xylobiose is incubated with the enzyme

M - products when xylotriose is incubated with the enzyme

G - glucose

A - arabinose

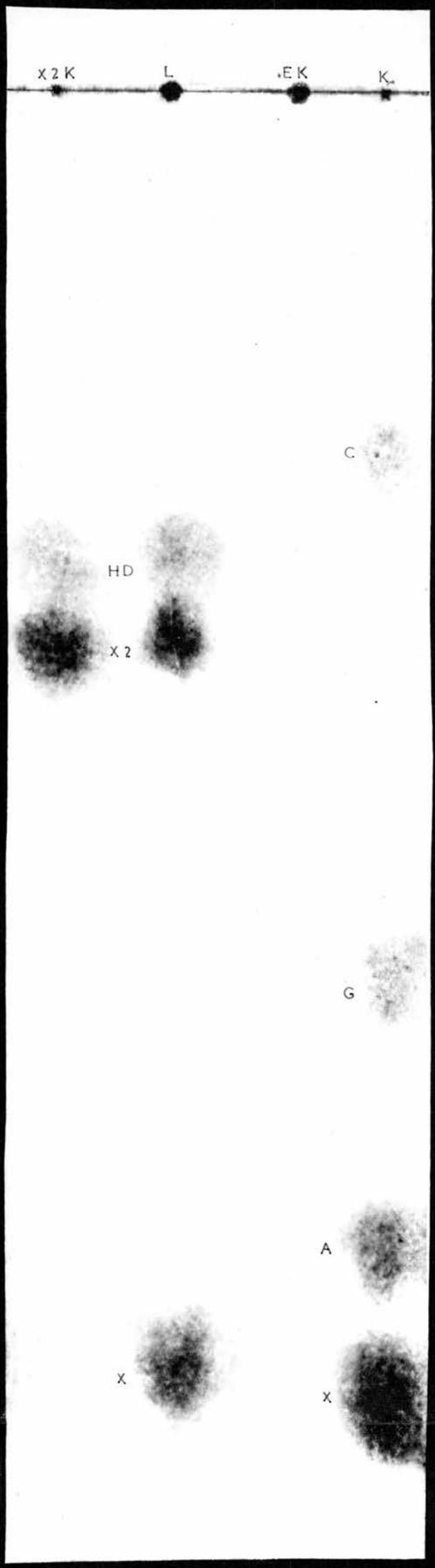
X - xylose

C - cellobiose

X2 - xylobiose

X3 - xylotriose

HD - hexosic disaccharide (contaminant of X2).


X2 K
L
EK
K
C
HD
X2
G
A
X
X

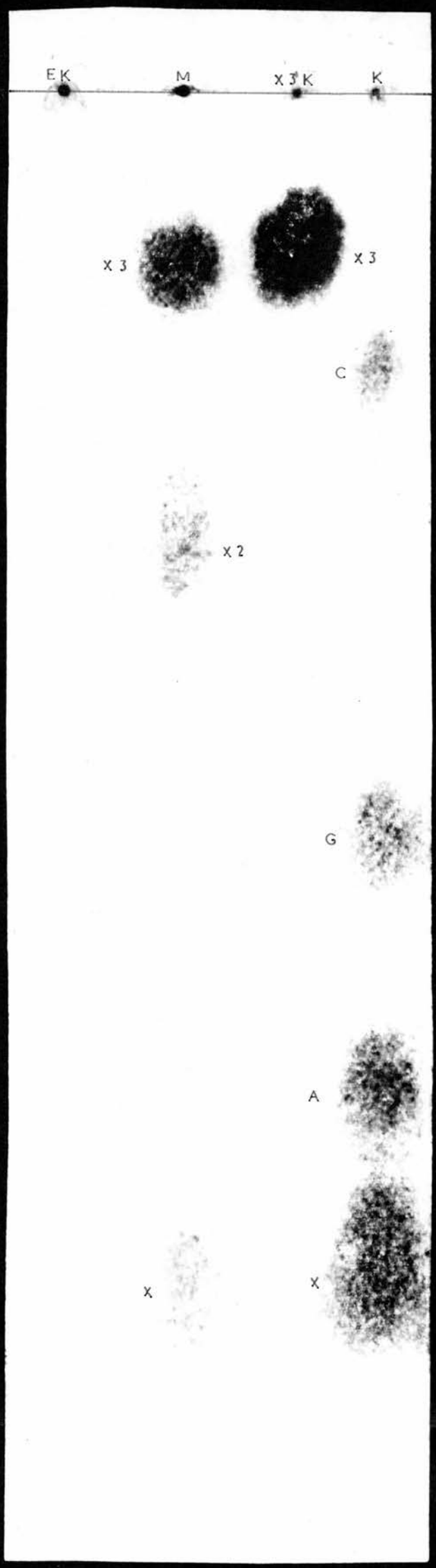

EK
M
X3 K
K
X3
X3
C
X2
G
A
X
X

TABLE V

Sugar and Oligosaccharide Production from Araboxylan by Chemically Treated Enzyme Preparations from Oats.

Treatment of Enzyme Solution[1]	Reagent		
	Calcium acetate	Phenylacetic acid	Phenylmercuric nitrate
Amount of reagent added (mg.)	40	100	100
Temperature (°C)	70	17	17
Time (hr.)	¼	5	3
Oligosaccharides on starting line	++	++	++
Higher oligosaccharides	-	-	±
Penta-oligosaccharides	±	+	+
Tetra-oligosaccharides	±	+	+
Tri-oligosaccharides	±	±	+
Xylobiose	-	-	±
Glucose	-	+	±
Arabinose	-	+	-
Xylose	-	-	-

[1] Each enzyme solution contained 40 mg. of the enzyme preparation from oats in 20 ml. water.

precipitated with 4 volumes of acetone and, after centrifugation, dissolved in 10 ml. water. Typical incubation mixtures were made using the appropriate volume of this enzyme solution. Qualitative results are also shown in Table V.

Heat inactivation: 20 mg. of an oats enzyme preparation in 0.2% solution was heated at 65°C. for 10 min. After cooling, a typical incubation mixture was made and incubated at 37°C. for 48 hr. The products were essentially the same as those obtained without heat treatment of the enzyme, but the amount of arabinose was considerably diminished. A small amount of xylose had been produced together with a trace of xylobiose. Oligosaccharides from the trisaccharide level upwards were also produced.

Action of an enzyme preparation from barley upon araban: Araban was prepared from sugar beet according to the method of Goodban and Owens (1957). Replacing the araboxylan substrate by the crude araban, a typical incubation mixture was set up, using a barley enzyme preparation (Series II). Although the identities of the products of enzyme action, other than arabinose, were not ascertained, it was clear that the araban was, in some measure, susceptible to attack by the barley enzyme preparation. The substrate, incubated under similar conditions, in the absence of the enzyme showed no degradation.

DISCUSSION

The amounts of arabinose and xylose liberated by the enzyme preparations from the common cereals when they were incubated with rye araboxylan (cf. Table III) are plotted against the corresponding periods of incubation in Figs. 2-6; those liberated by the barley enzyme from different samples of rye araboxylan (cf. Table IV) are plotted in Figs. 7-11. There are two features common to the 10 figures: Arabinose production is in all cases linear from the start; xylose production becomes linear after a variable period, dependent both upon the substrate and the enzyme.

It will be observed that there is a tendency for the arabinose figures to fall after reaching a level of 1000 µg. arabinose/5 ml. aliquot. A decrease in the rate of production of arabinose would be expected, for the reaction is an enzymic one, but the observed fall in the actual amount of arabinose is anomalous, and is at present being made the subject of further investigation. The observed fall, if not attributable to some inadequacy in the technique of estimation, could be accounted for by enzymic synthesis, but experimental results suggest that, under the conditions employed, the possibility of synthesis is remote. An alternative explanation would be in transarabinosylation, free arabinose units becoming

Figures 2 - 6.

Figures 2-6

Quantitative liberation of arabinose and xylose from rye araboxylan during its incubation with enzyme preparations from the five common cereals. The amount of the pentose in a 5 ml. aliquot of the incubation mixture is plotted against the time of the incubation.

x represents arabinose

o represents xylose

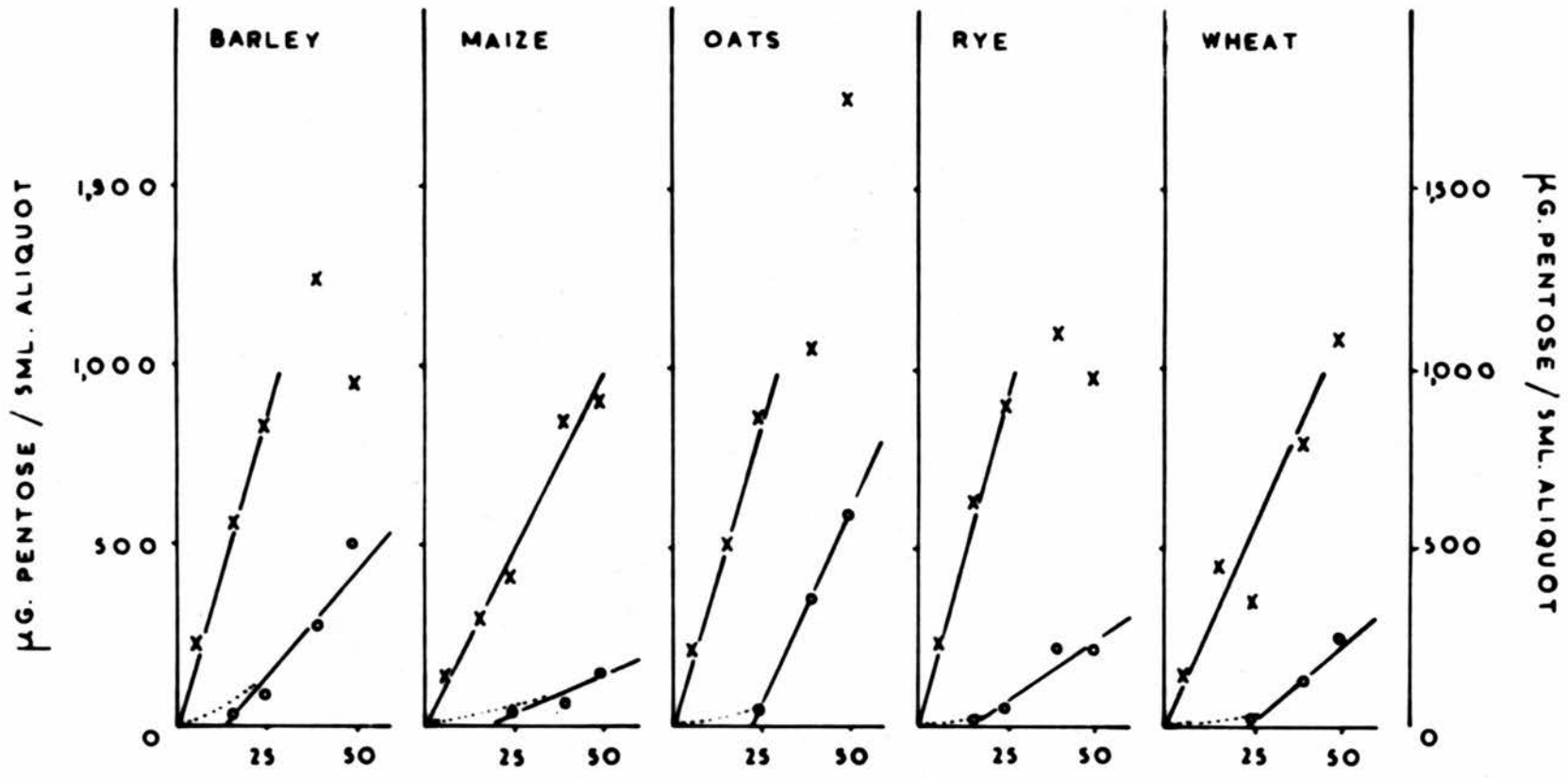


FIG. 2 FIG. 3 FIG. 4 FIG. 5 FIG. 6

Figures 7 - 11.

Figures 7-11

Quantitative liberation of arabinose and xylose from different samples of rye araboxylan during incubation with an enzyme preparation from barley. The substrates corresponding to Figs. 7, 8, 9, 10 and 11 are Rf (iii), Rg(iii), Rh, a 25 hr. "dextrin" and Rg (iii) at $\frac{1}{2}$ standard substrate concentration respectively (see Table IV). The amount of the pentose in a 5 ml. aliquot of the incubation mixture is plotted against the time of incubation.

x represents arabinose

o represents xylose

µG. PENTOSE / 5ML. ALIQUOT
1,500
1,000
500
0
25
50
TIME (HR.)
FIG.7
FIG.8
FIG.9
FIG.10
FIG.11

attached once again to the xylan backbone of either oligosaccharides or residual gum. Preece and Hobkirk (1954, 1955) have suggested that in wheat, at least, there may be some mechanism of transarabinosylation, but in this case arabinosyl side-chains, as opposed to free arabinose, which is to be considered in the present investigation, were thought to be involved in the transfer.

It is assumed that the substrates used are araboxylans of the type investigated by Aspinall and Sturgeon (1957), containing little or no free araban. Evidence for this assumption is provided by the above workers, whose method of preparation of the araboxylan from rye was similar to that used in the present investigation; the results, provided by methylation and periodate oxidation studies, failed to suggest the presence of free araban. Further, a comparison of Figs. 7-9 indicates that an increase in the arabinose/xylose ratio is reflected in a decrease in the rate of production of xylose. While it has been shown that the enzyme preparation from barley causes the liberation of arabinose from the araban prepared from sugar beet, the presence of araban in the araboxylan preparation Rg (iii), which has a high arabinose/xylose ratio, would be expected to have an additive effect on the amount of arabinose produced, but to have no effect upon xylose production.

An examination of Tables III and IV indicates a close relationship between the production of xylose and of xylobiose; it would appear that their formation is almost simultaneous. Xylobiose was eventually produced by the enzyme preparations from all five common cereals, whereas oligosaccharides from the trisaccharide level upwards were liberated later than xylobiose, in the presence of the barley and oats enzymes, and only in trace quantities, if at all, by the enzyme preparations from the other cereals in the 50 hr. incubation period investigated. It would appear, then, that the pattern of degradation is the same for all five common cereals, but that the balance of enzyme potentialities of each individual source may be reflected in quantitative differences. It is proposed that the degradation pattern may be explained on the basis of the following enzyme systems: (a) An endo-xylanase, responsible for diminution in the viscosity of araboxylan and for the production of oligosaccharides or 'dextrins' by progressive scission of the xylan backbone chain. (b) An arabinosidase, removing arabinosyl side-chains randomly from xylan backbone chains irrespective of their length. (c) An exo-xylanase, liberating free xylobiose from the non-reducing ends of xylan backbone chains. The results suggest - and this will be discussed in more detail later - that the removal of the arabinosyl

side-chains from the two terminal xylose residues is a necessary preliminary to exo-xylanase action.

(d) A xylobiase, liberating free xylose from xylobiose.

If it is accepted that the four enzyme systems detailed above are predominantly responsible for the changes observed, it is interesting to examine the results for endo-xylanase activity and for the production of arabinose, xylose, xylobiose and higher oligosaccharides by the enzyme preparations from the different cereals. For purposes of comparison, the endo-xylanase activities (as in Table II) and the gradients of the straight lines in Figs. 2-6, which correspond to the rates of production of arabinose and xylose, are recorded in Table VI. It should be emphasised that only the early stages of reaction are to be considered.

Barley and oats. The enzyme preparations from these cereals probably represent an evenly-balanced mixture of the four enzyme systems, such that any one system is not especially hindered by the inadequacy of another. The endo-xylanase produces many end-groups and many short chains and may thus permit the production of chromatographically detectable oligosaccharides after about 40 hr. incubation. The quick removal of arabinose allows exo-action, which may also eventually lead to the production of lower oligosaccharides. That oligosaccharide production is not entirely dependent upon endo-action is

TABLE VI

Comparison of Pentosanase Activities of Enzyme Preparations from Common Cereals.

Cereal	Endo-xylanase†	Rates of production of pentoses Ø		Production of oligosaccharides ⌘
		Arabinose	Xylose	
Barley	0.013	34.5	12.5	++
Maize	0.018	20.0	4.5	-
Oats	0.027	32.5	22.0	++
Rye	0.012	35.0	7.0	±
Wheat	0.007	22.0	8.5	-

† As in Table I.

Ø Increase in amount of pentose (µg.) liberated per hr. per 5 ml. aliquot.

⌘ Production of chromatographically detectable oligosaccharides, i.e. from tri- to penta-saccharide level, in the 50 hr. incubation period.

suggested by the capacity of the barley preparation to produce oligosaccharides as soon as, if not sooner than, the oats preparation, whereas the endo-activity of the barley preparation, as measured by viscosity diminution, is approximately half that of the oats preparation. Arabinosidase activities being approximately equal for the two preparations, it would appear that greater exo-xylanase activity of the barley preparation, as compared with that from oats, may compensate for the lower endo-activity of the former preparation, with regard to the production of chromatographically detectable oligosaccharides.

Rye. The preparation from rye is comparable with that from barley in rate of production of arabinose and in endo-xylanase activity. The contrasting features between the two preparations, however, are the low rate of xylose production and the absence of oligosaccharide production in the presence of the enzyme from rye. It would seem that the rye preparation has poor exo-activity. Chromatographic examination indicates that little xylobiose is present, even after 50 hr., and it is probable that it is split to xylose soon after its liberation by the exo-enzyme.

Maize. The outstanding feature of the experiments with the maize preparation is the lack of production of chromatographically detectable oligosaccharides or even

of xylobiose (which is present only in trace amount after incubation for 50 hr.) despite the high endo-activity of the enzyme preparation. It is certain that there is little exo-activity in the preparation; the arabinosidase is not so active as that in the barley, oats or rye preparations and the action of the exo-enzyme is probably hindered by the slow removal of arabinosyl side-chains from the terminal xylose residues of oligosaccharides.

Wheat. Although the patterns of production of arabinose and xylose by the wheat preparation are similar to those for maize, there is a striking difference in the endo-activities of the two preparations. It is apparent that the production of xylobiose by the wheat preparation is limited both by poor endo- and by poor arabinosidase-activity. Thus the action of the exo-enzyme, even if it were present in abundance, would be ineffectual, since few end-groups would be available, and of these very few would be suitable, for attack. Transarabinosylation of the type described by Preece and Hobkirk (1954, 1955) may in some measure compensate for poor arabinosidase activity by causing a transfer of arabinosyl units from terminal positions in the xylan chain to positions remote from the end.

Action of the barley enzyme on substrates Rf (iii), Rg (iii), Rh. The behaviour of the barley enzyme in the

presence of several substrates, illustrated by Figs. 7-9, is also capable of explanation on the basis of the four enzyme systems detailed above. The rate of production of arabinose is not much altered by a change in substrate, whereas the rate of production of xylose is apparently slower, the more arabinose there is present in the substrate.

Action of the barley enzyme on the 25 hr. 'dextrin'. The time of first observing xylose, xylobiose and higher oligosaccharides is earlier when the 25 hr. 'dextrin' (Fig. 10) is used as substrate, but the rate of xylose production is comparable with that observed with other substrates. The degraded substrate, which almost certainly contains a greater number of end-groups per unit mass than would a typical substrate, and which probably contains a higher proportion of free xylose residues in terminal positions, is more susceptible to exo-xylanase action than the typical substrate.

Effect of halving the substrate concentration. A comparison of Figs. 8 and 11 indicates that the rates of arabinose and xylose production are not much altered by the change in the concentration of the substrate Rg (iii). If it is assumed that an excess of the initial substrate is maintained in both experiments, throughout the incubation periods examined, little alteration in enzymic activity

would be expected.

Differential inactivation. From the results recorded in Table V, it would seem that calcium acetate causes the inactivation of the arabinosidase, of the exo-xylanase and, in part, of the endo-xylanase. Phenylacetic acid apparently inactivates the exo-xylanase since no xylobiose is produced; the endo-xylanase and arabinosidase are not destroyed. Phenylmercuric nitrate causes inactivation of arabinosidase but not of endo-xylanase. A trace of xylobiose is produced either by slight exo-action or by endo-action. The effects of the inhibitors upon xylobiase activity cannot be deduced from the results, for the appropriate substrate for the enzyme is not usually available. It must be emphasised that the absence of arabinosidase activity may completely preclude exo-xylanase action, it being assumed that the exo-enzyme requires the presence of two free (i.e. unattached to arabinose) xylose units at the chain end.

On the basis of the four enzyme systems postulated earlier, the action of the five enzyme preparations from the common cereals, and the behaviour of the barley enzyme with different substrates, are capable of explanation. That an arabinosidase and an endo-xylanase are present is undoubted; the presence of an exo-xylanase and of a xylobiase was assumed, as was the blocking action of

arabinosyl side-chains on terminal xylose residues; these assumptions require substantiation.

Blocking action by arabinosyl side-chains. Figs. 2-11 show that, while minor amounts of free xylose may be liberated in the early stages of the reaction, its production does not become rapid and linear until 3-5% of the total arabinose has been liberated in the free state. This suggests a blocking action by the arabinosyl side-chains. The two enzyme systems which may be affected by this action are (a) the endo-xylanase system and (b) the exo-xylanase system.

It is at present impossible to determine the nature of the distribution of arabinosyl side-chains along the xylan chain, and it is, therefore, assumed that the distribution is random, but whether this is so, or the arrangement is ordered, is of little consequence to the present argument.

In relation to (a), the effect of arabinosyl side-chains upon endo-xylanase action, it may be shown (See Appendix) that, if p is the proportion of xylose residues in a long chain not attached to arabinose (represented by -x-), and q is the proportion carrying arabinosyl side-chains (represented by $-\overset{a}{x}-$), then the proportion of -x- with $-\overset{a}{x}-$ on both sides is pq^2N, where N is the xylan backbone chain length, which, for purposes of argument,

will be taken as 100; similarly, the proportion of -x-x- with $-\overset{a}{x}-$ on both sides is p^2q^2N. Since p in a typical substrate is about 0.4, the arrangement $-\overset{a}{x}-x-\overset{a}{x}-$ may be expected to occur in 14.4% of the total arrangements, and $-\overset{a}{x}-x-x-\overset{a}{x}-$ in 5.9%; with elimination of 5% of the total arabinose, p becomes 0.43, and the corresponding probabilities 15.5 and 6.2% respectively. The differences appear to be too small to account for the relatively great change in behaviour. It seems improbable, therefore, that the arabinosyl side-chains have any significant influence on the initial disruption of the xylan chains.

It remains to consider the possibility of hindrance to exo-action by the arabinosyl side-chains. It might be argued that some xylobiose, and hence some xylose, would inevitably be produced from the start, and this is probably true, and is represented in Figs. 2-11 by curved lines from the zero-point; the linear production of xylose, however, is delayed. It has already been shown that in 14.4% of the total arrangements, $-\overset{a}{x}-x-\overset{a}{x}-$ may be expected to occur; thus the probability that $x-\overset{a}{x}-$ is terminal is small, and the probability that $x-x-\overset{a}{x}-$ is terminal is much smaller. Thus, if exo-action is dependent upon the availability of a structure of the type $x-x-\overset{a}{x}-$, it is to be expected that there will be a time-lag before sufficient substrate, in excess of the 'capacity' of the exo-enzyme,

is present; this condition is perhaps fulfilled at the 3-5% arabinose removal stage. At this stage, xylobiose is being produced in sufficient quantity to 'saturate' the xylobiase; hence the linear production of xylose begins.

Further evidence for a blocking action by arabinosyl side-chains is provided by Figs. 7-9; the percentage of the total arabinose which must be removed before xylose production becomes linear is not dependent upon the amount of arabinose in the substrate, but the rate at which xylose is produced is less, the more arabinose there is initially present; the additional arabinosyl side-chains in the 'arabinose-rich' substrate Rg (iii) will be distributed along the chain, and the proportion of these on terminal xylose residues will not be much altered; thus exo-action may commence equally readily, but will not proceed so quickly.

Evidence for the presence of an exo-xylanase and of a xylobiase. It may be argued that the action of a xylo-oligosaccharase which liberates free xylose from the non-reducing end of the xylan backbone chain could replace the combined action of the exo-xylanase and xylobiase. The production of oligosaccharides including xylobiose would then result from endo-action and prolonged xylo-oligosaccharase action. If this were so, the production

of the higher oligosaccharides and of xylobiose should be simultaneous, or higher oligosaccharides should be produced earlier than xylobiose; this is contradictory to the observed facts; xylobiose is in all cases of normal enzymes produced before higher oligosaccharides.

That a xylobiase exists has been demonstrated experimentally by the action of an oats enzyme preparation upon xylobiose, but whether its action is specific for a disaccharide, or it can also be responsible for the removal of xylose from xylotriose and from the chain-ends of higher oligosaccharides, is uncertain. The results of the differential inactivation of an oats enzyme preparation by phenylacetic acid serve as direct proof of the existence of an exo-xylanase, for neither xylobiose nor xylose is produced, whereas higher oligosaccharides are. The replacement of exo- and xylobiase-function by xylo-oligosaccharase action would fail to account for the observed absence of xylobiose, but would not preclude the joint action of all three enzyme systems, i.e. exo-xylanase and xylo-oligosaccharase action (which, in its action upon a disaccharide, would represent xylobiase action) in the uninhibited system of enzymes present in the original oats preparation.

It would appear, then, that there is competent evidence for an arabinosidase, an endo-xylanase, an exo-xylanase and a xylobiase. The mode of production of oligosaccharides

has already been discussed in relation to the enzyme preparations from barley, oats and maize. It may be concluded from the results with the maize enzyme - and those with the barley and oats preparations tend to confirm this - that endo-action alone is not responsible for the production of chromatographically detectable oligosaccharides, i.e. oligosaccharides from the tri- to the penta-saccharide level; exo-action is apparently, in some measure, necessary for their production, at least in the 50 hr. period examined. It may be that the endo-enzyme is selective, having preferential activity towards comparatively long xylan chains; fairly large oligosaccharides, which would not be detected chromatographically, would thus be liberated. (Analogous behaviour has been observed by Hopkins (1946) in the greater affinity of α-amylase for starch than for mixed dextrins; the enzymic reaction was found to become slower during the fission of dextrins for which the enzyme possessed ever smaller and smaller affinity). Exo-action would then, at least in the early stages of the reaction, be responsible for the production of the lower oligosaccharides, for, by the successive removal of xylobiose from the ends of the xylan chains of the higher oligosaccharides produced by endo-action, lower oligosaccharides would be formed, which could be detected chromatographically. Thus the earlier appearance of

oligosaccharides in the presence of the barley enzyme compared with the maize enzyme, despite the greater endo-activity of the latter, would be explained.

Comparison of the pentosanase system with the β-glucanase system. It is now possible to compare the enzyme systems responsible for the degradation of the two main groups of water-soluble non-starchy polysaccharides of cereal grains. In 1954a, Preece et al., using barley β-glucan, provided evidence for 3 types of action: (a) endo-β-glucanase action (b) exo-β-glucanase action, liberating cellobiose (c) cellobiase action. (The presence of an exo-β-glucanase system was evident, since the formation of cellobiose, unaccompanied by other oligosaccharides, was observed; this could not be accounted for by endo-attack alone.) It would appear that the modes of degradation of the two substrates, araboxylan and β-glucan, are analogous. In view of the structural similarities which exist between the two polysaccharides, the xylan backbone chain consisting entirely of 1:4-β-linkages and the β-glucan both 1:4- and 1:3-β-linkages, the possibility of enzymic hydrolysis of the common linkage by the same enzyme suggests itself. In relation to endo-action, the results of the experiments measuring the viscosity diminution of the two substrates, in the presence of enzyme preparations obtained during the

commercial malting of barley demonstrated the individual nature of the endo-xylanase and endo-β-glucanase systems; it is interesting, however, to note that those cereals which were found by Preece and Hoggan (1956) to yield active endo-β-glucanase preparations also yield active endo-xylanase preparations. Present results are inadequate to allow judgement upon a possible relationship between the exo-enzymes for the two substrates, or between xylobiase and cellobiase action.

The relation between cellulase and xylanase was investigated by Sørensen (1957) who concluded that some soil microorganisms are capable of producing an extracellular cellulase with some activity against xylan, and a xylanase with some activity against partly degraded cellulose. Bishop and Whitaker (1955) demonstrated the degradation of xylan by an enzyme preparation from the fungus Myrothecium verrucaria, a similar enzyme preparation having been found by Whitaker (1953) to possess cellulase activity; since it is impossible to ascertain the absolute purity and homogeneity of an enzyme preparation, however, it cannot be concluded that the xylanase and cellulase are identical.

Comparison of the cereal pentosanases with those from other sources. Lack of precise data regarding the structural features of many of the substrates used for

enzymic investigations has tended to confuse resulting information. Nevertheless, some general points emerge which indicate similarity between the modes of pentosan degradation by cereal enzymes and by those from other sources. Sørensen (1957) concluded, from his investigations with soil microorganisms, that there was an extracellular endo-xylanase responsible for the production of oligosaccharides; the shortest chain which could be attacked by the endo-xylanase was that of xylotriose; there was evidence to suggest that the central link of xylotetraose was more susceptible to enzymic attack than the terminal links - an indication of the probable presence of an exo-xylanase. In five out of six of the extracellular enzymes examined, no xylobiase activity was reported, but such activity was apparent in all six species if ground cell material replaced culture fluid as enzyme source. Using a substrate similar to that of Sørensen and an enzyme preparation from Myrothecium verrucaria, Bishop and Whitaker (1955) isolated six oligosaccharides which included xylobiose and five mixed oligosaccharides containing arabinose and xylose. Only trace amounts of xylose and arabinose were produced; the results suggest, therefore, that the preparation contained no arabinosidase or xylobiase, but only an endo-xylanase and, perhaps, an exo-xylanase.

It cannot be doubted that the present problem would be considerably simplified if a pure xylan substrate were found, for chromatographic examination of the hydrolysis products would almost certainly be facilitated in the absence of mixed arabinose-xylose oligosaccharides. Unfortunately, it has, as yet, been found impossible to prepare a water-soluble, arabinose-free xylan; as suggested by the Perlin (1951) hypothesis, the solubility of araboxylan decreases with decreasing proportions of arabinosyl side-chains, and the xylan becomes insoluble before arabinose removal is complete. Alternatively, it may become possible, by improved chromatographic procedure, to effect better separation of the mixed oligosaccharides. Nevertheless, the value of the chromatographic methods at present available cannot be underestimated, for, without them, the present investigation would have been impossible.

SUMMARY

1. Endo-xylanase activity was measured by the fall in the viscosity of a solution of rye araboxylan in the presence of cereal enzyme preparations. The validity of such measurements, in spite of contamination of the

substrate by a minor amount of β-glucan, was established. Evidence was provided for the individual existence of endo-xylanase and of endo-β-glucanase.

2. The amounts of arabinose and xylose liberated from rye araboxylan, by the action of enzyme preparations from the five common cereals, were measured. Arabinose production was linear from the start; xylose production became linear after 3-5% of the total arabinose of the substrate had been removed.

3. Evidence from 1. and 2. and from chromatographic investigations led to the conclusion that four enzyme systems were responsible for pentosan degradation:
(a) Endo-xylanase. (b) Arabinosidase, removing arabinosyl side-chains from xylan backbone chains. (c) Exo-xylanase, liberating xylobiose from the ends of xylan backbone chains. It appeared that the exo-xylanase action required the availability of at least two free (i.e. unattached to arabinose) terminal xylose residues.
(d) Xylobiase.

4. In addition to the monosaccharides arabinose and xylose, xylobiose, xylotriose and xylotetraose and mixed arabinose-xylose oligosaccharides, including a trisaccharide arabinosyl-xylosyl-xylose, have been recognised in the products of enzymic hydrolysis of araboxylan.

5. The five common cereals examined differed significantly in the balance of enzyme potentialities; oats and barley appeared to be rich in all four enzyme systems.

6. It appeared that the modes of action of the pentosanase system and of the β-glucanase system were analogous.

GENERAL DISCUSSION

Every research worker must, at some time, have been asked to establish the significance of his work by linking it to some practical application. While it is not unreasonable that one should attempt to justify some types of research in this way, it is often extremely difficult to do so truthfully and objectively, for much foresight is generally necessary to appreciate the implications of results and apply them to technical processes. Perhaps the present cereal investigation is nearer to economics than many other research topics, and yet it is impossible to relate the results directly to some future technical advance.

It was in connection with a practical problem - the loss which occurred in the manufacture of cereal starches due to the formation of a sludge - that Simpson (1954) undertook an enzymic investigation of pentosan degradation. Earlier, Clendenning and Wright (1950) had suggested that pentosan, in a strongly hydrated form, enveloped the starch granules with the formation of mucilaginous material known as the "squeegee" fraction of wheat flour; this accounted for considerable losses of starch during its separation from gluten. Simpson (loc. cit.) proposed that selective enzymatic hydrolysis of the pentosans should release the starch granules and named

Bacillus subtilis as a good source of an active pentosanase. It should be of interest to know whether some cereal enzyme, in particular that from wheat, would effect the necessary pentosan degradation, but, in view of the weak pentosanase activity exhibited by wheat enzyme preparations examined in the present investigation, it is doubtful whether their use would be advantageous; furthermore, the presence of amylases in the enzyme preparations is undoubted and starch degradation would almost certainly take place simultaneously with pentosan degradation.

Evidence for the importance of the water-soluble pentosans of wheat flour in determining the character of doughs used in bread-making has been provided by Pence *et al*. (1950), and although Kent-Jones and Amos (1957) failed to relate the total pentosan content of wheat flour with dough characters, the possibility that the physical properties of one type of pentosan are important cannot be ruled out. An inverse relationship between total pentosan and milling acceptability was found by Hale *et al*. (1953) when the pentosans were extracted from wheat with 2N hydrochloric acid at 25°C. Endospermic pentosans, and in particular xylan, probably in the form of araboxylan, were most important in controlling the relationship.

Many of the enzymic investigations regarding the

cereal hemicelluloses have related to barley and its behaviour during malting. Thus Preece and Mackenzie (1953) developed the autolytic technique used in Chapter 1 with the idea of making possible an assessment of malting quality of a barley by a comparatively short preliminary examination of the raw cereal. The method was shown to be of variable utility and served only to stress the impossibility of basing such a wide process as modification upon the behaviour of a single component of the barley corn. The changes undergone by hemicelluloses during the commercial malting of Ymer barley were investigated by Preece and Hoggan (1957), who demonstrated an increase in water-soluble β-glucan during the flooring period followed by a marked decrease during kilning. The soluble pentosan present followed a similar, though less pronounced pattern, but did not exhibit the final decrease; these results are in direct agreement with the endo-xylanase activities recorded in Chapter 4 for a series of enzyme preparations from Proctor barley during malting, and with the autolysis results of Chapter 1. Mechanical factors play an important part in pentosan solubilisation and the observed increase, during the flooring period, in soluble pentosan is attributable mainly to this factor, but also, in part, to an enzymic one which is of increased importance towards the end of the flooring

period. Mechanical solubilisation is no longer a major factor during kilning and pentosan enzymolysis remains relatively small, certainly in comparison with enzymatic degradation of β-glucan. Thus an appreciable amount of soluble pentosan is present in the final malt, although, as would be expected on account of the pentosanase characters examined in chapter 4, the viscosity of the final pentosan is considerably less than that in the original barley. The influence of the residual gums upon the viscosity of wort and beer has been investigated by Meredith et al. (1951), and it would appear that the pentosans rather than the glucans, which are in an extensively degraded condition in the final malt, contribute to wort viscosity.

Much attention seems to have been given to the detection of pentoses in wort, though why this topic is of interest to the brewer is not clear. Evidence is in general conflicting, but it would appear that the presence of free arabinose and xylose in malt (e.g. MacLeod et al., 1953; Hall et al., 1956) is not abnormal. It has been suggested (Fink, 1935) that synthetic mechanisms may account for the apparently transient appearance of the pentoses, and it is appropriate at this stage to consider the possibility of transarabinosylation for which an enzymic mechanism

has frequently been suggested (e.g. Preece and Hobkirk, 1954, 1955) although all the evidence available for such a mechanism is indirect. The problem is not an easy one, although if more rigorous methods of differential inactivation of enzymes were achieved, selection of the transglycosidase, if present, and recognition of its function, would be facilitated. An attractive method would be the use of radioactively labelled pentoses or pentosans; certainly the problem of decreased arabinose production in the later stages of enzyme action upon rye araboxylan (see chapter 4) would be capable of easy solution, if a type of transarabinosylation, involving free-arabinose transfer, were indeed responsible for the observed decrease.

The salient difficulty in all investigations involving the use of cereals is the impossibility of identifying quantitatively any property of one sample of a given cereal and the same property of another sample of the cereal, for a limitless number of factors is variable as, for example, the environment and conditions during growth and ripening, subsequent storage treatment, etc. In view of this, it is surprising that the order of the endo-xylanase activities for the 2 series of enzyme preparations from the five common cereals (see chapter 4) is the same for both series, the activity decreasing from the oats, through the maize, barley and rye, to the wheat

preparation. The autolytic technique which should, in the 4 hr. period applied, represent principally a measurement of endo-activity of an enzyme preparation, whether in its solubilizing or oligosaccharide-forming capacity, emphasises, in the different patterns of soluble material recovered (Preece and Aitken, 1953), the variable nature of the enzymic potentialities of different samples of barley. The results of the investigations of chapter 1, in which an enzyme preparation from Ymer barley caused some slight solubilization of the hemicelluloses of barley grain, but failed to produce chromatographically detectable pento-oligosaccharides from the soluble material within 24 hr., are to be compared with those in chapter 4, where, using an enzyme preparation, also from Ymer barley, arabinose was formed from the start and, after 25 hr., oligosaccharides were produced, including xylose and xylobiose which were present even after 16 hr. It has also been found that different samples of oats yield enzyme preparations with differing tendencies to liberate pento-oligosaccharides from araboxylan; thus it was occasionally, although infrequently, possible to prepare pure xylotriose or xylotetraose, uncontaminated by arabinose-containing oligosaccharides, without the application of purification procedures. Linked with these observations was the apparent increase in the

tendency of an oats preparation to yield oligosaccharides after the dry enzyme had been left standing at room temperature for some months. It seems that the arabinosidase, xylobiase and probably the exo-xylanase are denatured slowly, and the endo-xylanase, which is generally more resistant to destruction (cf. differential inactivation experiments of chapter 4) remains, and allows the formation of oligosaccharides which are not so quickly degraded to arabinose and xylose as by enzyme preparations which have been preserved in a refrigerator. Similar destruction of enzymic activity was demonstrated by Sørensen (1953), who observed that an enzyme preparation from Chaetomium globosum lost its xylobiase activity when stored for a long period. The conclusion seems well-founded that the balance of the four enzyme systems (i.e. endo-xylanase, arabinosidase, exo-xylanase and xylobiase, which are together mainly responsible for pentosan degradation) in enzyme preparations from a particular cereal is fairly constant, although abnormalities may exist which alter the pattern of enzymic degradation; in general, preparations from the five common cereals contain the same enzyme systems but in different relative amounts.

It is interesting to note that maize is anomalous in many respects. Firstly, it contains very small amounts of pentosan material, a feature which may be

responsible for the popularity of maize as a source of starch, for interference of the type already discussed (Clendenning and Wright, loc. cit.) in the absence of large quantities of pentosans, is unlikely. Secondly, maize contains large quantities of a dextrorotatory glucan whose nature has already been discussed in chapter 2. Thirdly, in their enzymic potentialities maize preparations are outstanding for their high endo-activities both towards β-glucan and towards araboxylan; in contrast to this are low exo-β-glucanase activity and low capacity for producing free xylose. The observed failure to produce pento-oligosaccharides readily, despite high endo-xylanase activity, was a further atypical characteristic of the enzyme preparation from maize.

The pattern of pentosan degradation, at least in the presence of enzymes from barley, from the insoluble hemicellulosic stage to the monosaccharide level may be traced by considering together the results of the autolysis experiments and those of the enzymolysis of rye araboxylan. The first stage, when the grain becomes wet, is penetration of the solvent, which gradually dissolves accessible water-soluble pentosan; this is followed by enzymic hydrolysis of components of the grain other than pentosan, this action liberating less accessible pentosans which become dissolved. The final process in this stage is a very

slow dissolution of insoluble material; this may take place, in part, by an enzymic mechanism, but it is certain that mechanical factors also operate. Soluble pentosan - and it is now possible to consider only araboxylan - is at once subject to endo-attack which decreases the viscosity of the material and causes the liberation of a large number of end-groups; at the same time, arabinose is liberated from the araboxylan. After a variable period, xylobiose and xylose are produced and finally pento-oligosaccharides. The results suggested that an exo-xylanase was responsible for the production of xylobiose from the ends of xylan backbone chains, and that a xylobiase was partly, if not wholly, responsible for the liberation of free xylose.

As a result of the work of MacWilliam and Harris (1957), it seemed that the laminarinase system, like the β-glucanase system, consisted of both endo- and exo-components. It is probable, then, that there is a standard mode of attack by the polysaccharases, enzymolysis of starch, of β-glucan and of araboxylan being analogous. Thus each system is known to consist of an enzyme capable of rupturing at random the internal linkages of the polysaccharide molecule (cf. α-amylase, endo-β-glucanase, endo-xylanase); this action is accompanied by that of a component which splits off disaccharide units from the chain ends (cf. β-amylase,

exo-β-glucanase, exo-xylanase), whilst the disaccharides are finally hydrolysed by the corresponding glycosidases, namely maltase in starch degradation, cellobiase and laminaribiase in β-glucan breakdown, and xylobiase in xylan degradation.

It is certain that greater enzymic purification is desirable in future investigations. Preece and Hoggan (1956) obtained enzyme preparations of increased activity by carefully controlling the pH of the extraction medium and effecting ammonium sulphate fractionation at an optimum pH; such methods were found to increase the endo-xylanase activity of enzyme preparations slightly, but not significantly. It seems that the enzymic purification required will not be effected satisfactorily by chemical means, and it is probable that physico-chemical methods, such as that of MacWilliam and Harris (1957) will be required. These workers used gradient elution from an alumina column with phosphate-citric acid buffer, and separated a laminaribiase from a cellobiase, thereby ascertaining the specificity of β-glycosidases. Paper electrophoresis of enzymes has been applied by several workers and has generally indicated the lack of homogeneity of enzyme preparations but has, in most cases, failed to effect clear-cut separation of enzyme systems. Jermyn (1952) has demonstrated the presence of at least 8 active

components in a β-glucosidase preparation from Aspergillus oryzae. Sørensen (1957) applied the electrophoretic method to an extracellular xylanase preparation from Streptomyces and obtained two components which were active against xylan yielding, as reaction products, xylose, arabinose, xylobiose, xylotriose, and xylotetraose. It may be that column electrophoresis of enzyme preparations will effect their purification and yield larger amounts of the enzymes than are available by paper electrophoresis. This method has already been applied by Cooper and Pollock (1957) to a β-amylase concentrate from malt syrup. Several fractions were obtained which possessed β-amylase activity but which differed in electrophoretic mobility. The complexity of the problem was emphasised by these results which indicated that β-amylase activity was associated with substances of different molecular weights.

Linked to the problem of enzymic purification is that of substrate purification, for without the latter, detailed investigation of the former is profitless. A qualitative survey of enzyme character may be deduced from the use of a mixed substrate, but quantitative investigations and standardisation of enzymic activity are seriously hampered, if not impossible, when the substrate is not pure. Although the difficulty of

substrate impurity was overcome in the present investigation by the use of the ribose marker, the procedure was necessarily more tedious than would be a direct measurement of reducing group liberation. It would, therefore, be desirable to study the enzymolysis of a pure xylan, a pure araban, and of a pure araboxylan. Knowledge of the action of the cereal enzymes upon xylobiose, xylotriose and higher pento-oligosaccharides would also be useful.

There can be little doubt that the problem of the enzymatic degradation of pentosan, like that of β-glucan, is far from complete solution. Perhaps the most interesting question to be solved is the mode of formation of oligosaccharides, for the present results have indicated - contrary to what was previously believed - that endo-action is not entirely responsible for the early production of, at least, the lower oligosaccharides. Examination of the structures of such oligosaccharides (again difficult in practice for the amounts available are small and separation is, in any case, far from easy) may shed light upon their mode of formation. Most promising amongst the newer methods available for such structural investigation is that of electrophoresis, and it is to be hoped that electrophoretic techniques may be developed which will facilitate the separation, not only of the enzymes in cereals, but also of the products formed by the interaction of the enzymes with the corresponding substrates.

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APPENDIX

(i)

Suppose there are N lamps in a row; pN are white (W) and qN are red (R) where $p + q = 1$. It is required to find the most probable distribution of runs of W (with R on either side), if the arrangement is random.

$$\text{Total number of arrangements} = \frac{N!}{(pN)!(qN)!}$$

Add one R lamp to extreme right. As this is always present, it will not alter number of arrangements.

Total lamps now $N + 1$, pN are W, $qN + 1$ are R. To each red lamp attach the rW lamps immediately to its left, forming a parcel of $r + 1$ lamps consisting of rW in a row terminated by one R on right. A parcel of one lamp is a single R with no W on its immediate left. Form the sequence $u = \{u_1, u_2, u_3, u_4, \text{---------}\}$ where u_r is the number of parcels of r lamps. There is one sequence for each arrangement of the lamps, though a number of different arrangements may have the same sequence u.

$$\sum_r u_r = qN + 1$$
$$\sum_r r u_r = N + 1$$

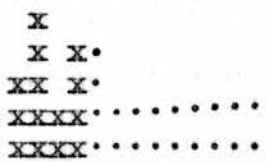
Number of arrangements that have a given sequence u is

$$\frac{(qN + 1)!}{(u_1)!(u_2)!(u_3)!\text{-------}} = \frac{(qN + 1)!}{\prod_r (u_r)!}$$

Probability of u is thus $\dfrac{(qN + 1)!(pN)!(qN)!}{N! \prod (u_r)!}$

The most probable sequence is obtained by minimising $K = \Pi(u_r)!$ subject to $\sum_r u_r = qN + 1$, $\sum_r r u_r = N + 1$.

Now form the discrete graph of u by stacking u_r particles vertically in the r^{th} place, e.g. the graph of $\{3, 5, 2, 4, \text{--------}\}$ would be



Our problem becomes the problem of minimising the product of the factorials of the heights, subject to the total number of particles and the r-position of their C.G. remaining invariant. The first condition means that any particle removed from one column must be placed on another. The second condition means that for every particle moved one place to the right, another particle must be moved one place to left. We move particles only from the tops of columns.

Suppose a particle is moved from a height y to a height z. The effect on K is to replace (y!) by (y - 1)! and to replace (z - 1)! by z!. K is hence multiplied by $\frac{z}{y}$, and is hence reduced by a downward movement of a particle. We reduce K by simultaneously moving one particle one place to the right, and another one place to the left, in such a way that the product of the two multiplying factors is less than 1. One motion will improve K and undoing this motion will bring K back to its previous value.

Hence there will usually be one way of improving K. This process will continue until over the whole graph the multiplying factor for one step to the right (which is the reciprocal of the multiplying factor for one step to the left) is constant, i.e. $\frac{u_{r+1}}{u_r} = c$ where c is a constant. $u_r = u_1 c^{r-1}$

$$\sum u_r = \sum u_1 c^{r-1} = \frac{u_1}{1 - c} = qN \quad (N \text{ is large})$$

$$\sum r u_r = \sum u_1 r c^{r-1} = u_1 \frac{d}{dc} \sum c^r$$

$$= u_1 \frac{d}{dc} \frac{c}{1 - c} = \frac{u_1}{(1 - c)^2} = N$$

$$1 - c = q \quad \therefore c = p$$

$$u_1 = q^2 N$$

$\therefore$ Most probable u is

$$\{q^2N,\ pq^2N,\ p^2q^2N,\ p^3q^2N,\ \text{-------}\}$$

Hence most probable distribution of runs of W is

pq^2N single W

p^2q^2N double W

p^3q^2N triple W

---

p^rq^2N runs of rW together.

NOTE A free xylose unit (represented by -x-) in chapter 4 corresponds to a white lamp. A xylose unit attached to arabinose (represented by $-\overset{a}{x}-$) corresponds to a red lamp.

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